

Molecular investigation of genetic factors associated with insulin resistance and obesity in a South African population

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes three (3) original papers published in peer reviewed journals and one (1) unpublished work. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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Date:

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“Have I not commanded you? Be strong and courageous. Do not be afraid; do not be discouraged, for the LORD your God will be with you wherever you go.” Joshua 1: verse 9.

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“Het EK jou nie beveel nie? Wees sterk en vol moed. Wees nie bevrees of verskrik nie, want die HERE jou God is met jou oral waar jy heengaan.” Josua 1: Vers 9.

Dedication

For Pa, Ma Pat and my big sister, Ashlene ☺

“It seems impossible until it is done” TYJ

Preface

This sub-study forms part of a larger study, the Bellville South Africa Study, in which risk factors for type 2 diabetes mellitus and cardiovascular disease were investigated in the mixed-ancestry population of Bellville South, South Africa.

A. Title of the larger study (The Bellville South Africa Study):

This is an epidemiological study that aims to elucidate obesity, diabetes, hypertension and the metabolic syndrome in adults from urban communities of the Bellville South, Western Cape, South Africa.

A.1 Ethics reference: Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee: CPUT/HWS-REC 2008/002 and CPUT/HWS-REC 2010

A.2 Background:

Recent data have shown that the prevalence of type 2 diabetes mellitus in the mixed-ancestry (coloured) population (26,3%) has more than doubled within the last decade. Moreover, about 18,1% of people with the disease are not aware of their condition and are therefore not receiving interventions with proven benefits for the adverse health consequences of diabetes. The increasing diabetes figures around the world are largely attributed to environmental changes that promote the adoption of unhealthy behaviour, resulting in the development of obesity. This in turn, is implicated in insulin resistance and inflammation and the subsequent progression to diabetes and cardiovascular disease.

A.3 Selected published articles from The Bellville South Africa Study:

Erasmus, R.T., Soita, D.J., Hassan, M.S., Blanco Blanco, E., Vergotine, Z., Kengne, A.P., Matsha, T.E. 2012. High prevalence of diabetes mellitus and metabolic syndrome in a South African mixed ancestry population: The Bellville-South Africa study - baseline data. *S. Afr. Med. J.*, 102:841-844.

Matsha, T.E., Hassan, M.S., Kidd, M., Erasmus, R.T. 2012. The 30-year cardiovascular risk profile of South Africans with diagnosed diabetes, undiagnosed diabetes, pre-diabetes or normoglycaemia: the Bellville, South Africa pilot study. *Cardiovasc. J. Afr.*, 23:5-11.

Matsha, T.E., Soita, D.J., Hassan, M.S., Hon, G.M., Yako, Y.Y., Kengne, A.P., Erasmus, R.T. 2013. Three-year's changes in glucose tolerance status in the Bellville South cohort: rates and phenotypes associated with progression. *Diabetes Res. Clin. Pract.*, 99:223-230.

Matsha, T.E., Hassan, M.S., Hon, G.M., Soita, D.J., Kengne, A.P., Erasmus, R.T. 2013. Derivation and validation of a waist circumference optimal cutoff for diagnosing metabolic syndrome in a South African mixed ancestry population. *Int. J. Cardiol.*, 168:2954-2955.

B. The sub-study investigated in this thesis:

Molecular investigation of genetic factors associated with insulin resistance and obesity in a South African population

B.1 Ethics reference number: Research Ethics Committee of Stellenbosch University: HREC Ref. No: N09/05/146

B.2 Background to the sub-study:

The aetiology of insulin resistance is complex and influenced by both environmental and genetic factors. In the present study, the focus is on the genetic factors that may predispose individuals to insulin resistance, subsequent type 2 diabetes mellitus and cardiovascular disease. The independent and joint effects of specific variants in genes associated with insulin resistance and diabetes were investigated, in order to enable a better understanding of the molecular and pathophysiological basis that may contribute to insulin resistance within the mixed-ancestry study population. Furthermore, insulin resistance is associated with both type 2 diabetes mellitus and cardiovascular disease, in which hyperinsulinaemia leads to insulin resistance. In addition, hyperinsulinaemia worsens the state of subclinical cardiovascular disease/atherosclerosis in type 2 diabetes mellitus, the major cause of mortality in diabetes subjects. Based on this evidence, the focus of the present study was to investigate whether indices of insulin resistance aid in the stratification of subclinical cardiovascular disease/atherosclerosis risk in mixed-ancestry South Africans.

B.3 Dissertation presentation:

This dissertation was written according to the prescribed guidelines of the Stellenbosch University. The dissertation is presented as an ‘article format thesis’. The norm is that at least three journal articles, with the candidate as the major contributor, must be included. At least two of these articles must be already published. Technical presentation of the thesis must follow the guidelines as stipulated, that is: the ‘article format thesis’ requires that the text of the articles must be retyped for inclusion in the dissertation according to standard guidelines. It is not acceptable to insert copies or off-prints of published articles directly into the dissertation.

B.3.1 Published articles emanating from this thesis:

Vergotine, Z., Yako, Y.Y., Kengne, A.P., Erasmus, R.T., Matsha, T.E. 2014. Proliferator-activated receptor gamma Pro12Ala interacts with the insulin receptor substrate-1 Gly972Arg and increase the risk of insulin resistance and diabetes in the mixed ancestry population from South Africa. *BMC Genetics*, 15:10.

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3900266/> [2014, 25 January].

Vergotine, Z., Yako, Y.Y., Kengne, A.P., Erasmus, R.T., Matsha, T.E. 2014. No evidence for association of insulin receptor substrate 1 Gly972Arg variant with type 2 diabetes in a mixed ancestry population in South African. *S. Afr. Med. J.*, 104:420-423.

<http://www.samj.org.za/index.php/samj/article/view/7419> [2014, 1 June].

Vergotine, Z., Yako, Y.Y., Kengne, A.P., Erasmus, R.T., Matsha, T.E. 2014. Rare mutations of peroxisome proliferator-activated receptor gamma: Frequencies and relationship with insulin resistance and diabetes risk in the mixed ancestry population from South Africa. *Int J Endocrinol.*, 2014:187985.

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4150434/> [2014, 12 September].

B.3.2 Non-accredited journal abstract published

Vergotine, Z., Erasmus, R.T., Hassan, M.S., Matsha, T.E. 2010. Prevalence of metabolic syndrome associated with glucose tolerance and body mass index in a mixed ancestry population of South Africa. *Journal of Nutrigenetics and Nutrigenomics*, 3:49–124.

B.3.3 Unpublished article emanating from this thesis

Vergotine, Z., Yako, Y.Y., Kengne, A.P., Erasmus, R.T., Matsha, T.E. 2014. Indices of insulin resistance and markers of sub-clinical cardiovascular disease/atherosclerosis. (Manuscript draft).

B.3.4 Conference presentations:

International

Vergotine, Z., Kengne, A.P., Pillay, T.S., Erasmus, R.T., Matsha, T.E. Indices of insulin resistance and markers of sub-clinical cardiovascular disease in mixed-ancestry South Africans. In the *International Federation of Clinical Chemistry and Laboratory Medicine, WorldLab Congress*, 2014 June 22-26, Istanbul: Turkey.

Vergotine, Z., Yako, Y.Y., Hon, G.M., Erasmus, R.T., Kengne, A.P., Matsha, T.E. Gly972Arg variant of the insulin receptor substrate-1 gene is not associated with type 2 diabetes, HOMA-IR and obesity in the mixed ancestry population of South Africa. *Proceedings of the 13th Asia-Pacific Federation for Clinical Biochemistry and Laboratory Medicine Congress*, 2013 October 27-30, Bali.

Vergotine, Z., Yako, Y.Y., Hassan, M.S., Erasmus, R.T., Matsha, T.E. Low frequency of Ala allele in the PPAR gamma2 in subjects at high risk of diabetes mellitus from South Africa. *World Diabetes Congress: organised by the International Diabetes Federation*, 2011 December 4-8, Dubai.

Vergotine, Z., Matsha, T.E., Hassan, M.S., Erasmus, R.T. Prevalence of metabolic syndrome associated with glucose tolerance and body mass index in a mixed ancestry population of South Africa. *Proceedings of the IV Congress of the International Society of Nutrigenetics/Nutrigenomics*, 2010 November 17-20, Pamplona: Spain.

National

Vergotine, Z., Yako, Y.Y., Hassan, M.S., Hon, D., Erasmus, R.T., Matsha, T.E. Insulin receptor substrate 1 sequence variants and type 2 diabetes in the mixed ancestry population of South Africa. *Pathpoint Congress*, 2012 September 28-30, Cape Town.

Vergotine, Z., Matsha, T.E., Hassan, M.S., Erasmus, R.T. The prevalence of Ala allele in PPAR-y2 in a mixed ancestry population of South Africa. *Laboratory Medicine Congress*, 2011 August 31-September 4, Sandton: Johannesburg.

Vergotine, Z., Matsha, T.E., Hassan, M.S., Erasmus, R.T. Measures of adiposity associated with fasting blood glucose and insulin levels in a population of South Africa. *Physiology Society South Africa Conference*, 2010 September 27-29, East London.

Vergotine, Z., Matsha, T.E., Hassan, M.S., Erasmus, R.T. Metabolic syndrome and glucose tolerance in an urban community from Cape Town, South Africa. *SAACB, Pathvine Congress*, 2010 September 2-5, Somerset West: Cape Town.

Abstract

Background: The aetiopathogenesis of type 2 diabetes and the associated insulin resistance have been shown to have a strong genetic basis. Several genetic variants of the peroxisome proliferator-activated receptor gamma (*PPARG*) and the insulin receptor substrate (*IRS*) 1 genes have been associated with the metabolic states of obesity, insulin resistance and type 2 diabetes in Caucasian populations. Furthermore, insulin resistance is strongly associated with diabetes and subsequent cardiovascular disease. These are increasingly common in low- to middle -income countries, including South Africa. Limited information is currently available regarding genetic associations with insulin resistance in African populations.

Objectives: (1) To identify subjects with insulin resistance and determine the frequencies of the single nucleotide polymorphisms in the *PPARG* and *IRS1* genes and examine the associated risk of insulin resistance and type 2 diabetes mellitus in a mixed-ancestry South African population. (2) To investigate the relationship between indices of insulin resistance and carotid intima media thickness, a marker of subclinical cardiovascular disease/atherosclerosis.

Methods: A total of 856 (235 males) mixed-ancestry adults drawn from an urban community of Bellville South, Cape Town were genotyped for *PPARG* Pro12Ala (rs1801282, G>C), Pro115Gln (rs1800571, G>T), Val290Met (rs72551362, G>A), Phe388Leu (rs72551363, T>A), Arg397Cys (rs72551364, C>T), His449His (rs3856806, C>T) and *IRS1* Gly972Arg (rs 1801278, G>A). The oral glucose tolerance test was performed and cardiometabolic risk factors measured. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance, the homeostasis model assessment of functional beta-cells, the quantitative insulin-sensitivity check index, the fasting insulin resistance index and the glucose/insulin ratio. Carotid intima media thickness was measured in longitudinal section at the far wall of the distal common carotid arteries, 2 cm from the bifurcation, at three consecutive end-points, 5-10 mm apart.

Results: The genotype frequencies of *PPARG* Pro12Ala, *IRS1* Gly972Arg and *PPARG* His449His were 10,4%, 7,7% and 23,8% respectively. No mutations were found for *PPARG* Pro115Gln, Val290Met, Phe388Leu and Arg397Cys. In a model containing both *PPARG* Pro12Ala and *IRS1* Gly972Arg alleles and their interaction term, the presence of the *PPARG* Pro12 resulted in a 64% risk of prevalent type 2 diabetes mellitus and was associated with higher 2 hour post-OGTT insulin levels in subjects with normoglycaemia. The *PPARG* Pro12 was associated with insulin resistance and interacted with *IRS1* Gly972Arg, increasing the risk of type 2 diabetes mellitus. The *PPARG* His449His allele T frequency was about 14% and in an additive genetic model significantly reduced the risk of diabetes by 44%. After adjustment for age, gender, body mass index and diabetes status, the fasting plasma glucose ($\beta=0,087$; $p=0,042$) and glucose/insulin ratio ($\beta=0,026$; $p=0,026$) were associated with carotid intima media thickness. However, the effect on the overall model performance was marginal, $R^2<29,7\%$.

Conclusion: The *PPARG* Pro12 was associated with insulin resistance and showed a gene-gene interaction with the unfavorable polymorphism *IRS1* Gly972Arg, leading to an increased risk of type 2 diabetes mellitus. In contrast, the *PPARG* His449His T allele showed a protective effect against the risk of developing diabetes. Furthermore, indices of insulin resistance such as homeostatis model assessment of insulin resistance, quantitative insulin-sensitivity check index, fasting insulin resistance index and the glucose/insulin ratio were weakly associated with carotid intima media thickness in the risk stratification of cardiovascular disease in this population.

Opsomming

Agtergrond: Die sterk genetiese grondslag van die etiopatogenese van tipe 2 diabetes en verwante insulienweerstand is reeds bevestig. Verskeie genetiese variante van die peroksisoomprolifererende geaktiveerde reseptor gamma (*PPARG*) en die insulienreseptor substraat (*IRS*)1 gene hou verband met die metaboliese sindroom van vetsug, insulienweerstand en tipe 2 diabetes in Kaukasiese bevolkings. Verder word insulienweerstand sterk verbind met gevolglike kardiovaskulêre siekte in diabetes mellitus. Laasgenoemde toestande neem toe in lae- en middelinkomste lande, Suid Afrika ingesluit. Beperkte inligting is tans beskikbaar aangaande die genetiese verwantskappe van insulienweerstand in Afrika bevolkings.

Doel: (1) Om proefpersone met insulienweerstand te identifiseer, die voorkoms van enkelnukleotiedpolimorfismes in *PPARG* en *IRS1* gene te bepaal en die verwante risiko vir insulienweerstand en tipe 2 diabetes in 'n Suid-Afrikaanse bevolking van gemengde herkoms te ondersoek. (2) Om die verwantskappe tussen insulienweerstandsindeks en karotis intima media dikte as 'n merker van subkliniese kardiovaskulêre siekte/aterosklerose te bepaal.

Metode: 'n Steekproef van 856 (235 mans) volwassenes van gemengde herkoms uit 'n stedelike gemeenskap in Bellville Suid, Kaapstad, is gegenotipeer vir *PPARG* Pro12Ala (rs1801282, G>C), Pro115Gln (rs1800571, G>T), Val290Met (rs72551362, G>A), Phe388Leu (rs72551363, T>A), Arg397Cys (rs72551364, C>T), His449His (rs3856806, C>T) en *IRS1* Gly972Arg (rs 1801278, G>A). Orale glukosetoleransie toetse is uitgevoer en kardiometaboliese risikofaktore is gemeet. Insulienweerstand is geraam deur die homeostasemodel assessering vir insulienweerstand, die homeostasemodel assessering van funksionale betaselle, die kwantitatiewe insulien sensitiviteit kontroleerindeks, die vastende insulienweerstandsindeks en glukose/insulien verhouding. Carotids intima media dikte is gemeet in longitudinale snit teen die verre wand van die distale gemene karotis arteries, 2 cm vanaf die bifurkasie, by drie opeenvolgende eindpunte, 5-10 mm uit mekaar.

Resultate: Die voorkoms van *PPARG* Pro12Ala, *IRS1* Gly972Arg and *PPARG* His449His genotipes was onderskeidelik 10,4%, 7,7% en 23,8%. Geen mutasies van *PPARG* Pro115Gln, Val290Met, Phe388Leu of Arg379Cys is gevind nie. In 'n model wat beide die *PPARG* Pro12Ala en *IRS1* Gly972Arg allele en hul interaksieterm bevat het, het die teenwoordigheid van *PPARG* Pro12 gelei tot 64% risiko vir tipe 2 diabetes en verband gehou met verhoogde 2-uur post-OGTT insulienvlakke in studiepersone met normoglisemie. 'n Verband is aangedui tussen *PPARG* Pro12 en insulienweerstand en daar was interaksie met *IRS1* Gly972Arg wat die risiko vir tipe 2 diabetes verhoog. Die *PPARG* His449His T-allel se voorkoms was ongeveer 14% en het die diabetesrisiko met 44% verlaag in 'n saamgestelde genetiese model. Na aanpassings vir ouderdom, geslag, liggaamsmassa indeks en diabetes status, is 'n verband getoon tussen beide vastende plasmaglukose ($\beta=0,087$; $p=0,042$) en glukose/insulienverhouding ($\beta=0,026$; $p=0,026$), en karotis

intima media dikte. Die uitwerking op die gehele model was egter gering, $R^2 < 29,7\%$.

Gevolgtrekking: 'n Verwantskap is getoon tussen *PPARG* Pro12 en insulienweerstand en 'n geen-geen interaksie het voorgekom met die ongunstige *IRS1* Gly972Arg polimorfisme wat lei tot verhoogde risiko vir tipe 2 diabetes. Die *PPARG* His449His T-allel het in teenstelling 'n beskermende uitwerking getoon teen die risiko vir diabetes. Verder was daar 'n swak verband tussen indekse van insulienweerstand soos homeostasemodel assessering, die kwantitatiewe insuliensensitiwiteit kontroleerindeks, die vastende insulienweerstandsindeks en glukose/insulien verhouding, en karotis intima media dikte vir risikogroepering vir kardiovaskulêre siekte in hierdie bevolking.

Outline of the dissertation

This dissertation is a compilation of four studies regarding genetic factors associated with insulin resistance (IR) and type 2 diabetes mellitus (T2DM), with focus on the peroxisome proliferator-activated receptor gamma (*PPARG*) and insulin receptor substrate (*IRS*)1 genes, as well as the use of IR indices in cardiovascular disease (CVD) risk stratification. The dissertation is arranged in a series of six chapters as follows:

Chapter 1: Provides an overview of the literature related to the condition defined as IR based on the molecular pathway, pathogenesis, aetiology, associated disease conditions with IR and CVD, various assessment methods of insulin resistance/sensitivity (IR/S) and subclinical atherosclerosis, epidemiology and the genes associated with IR.

Chapter 2: Presents a detailed research methodology.

Chapter 3: Examines the independent and joint effects of *PPARG* Pro12Ala and *IRS*1 Gly972Arg variants on markers of IR and T2DM. The findings of this chapter combine two papers that were published in the South African Medical Journal and BioMedical Central Genetics Journal in 2014.

Chapter 4: Examines the association between cardiometabolic traits, *PPARG* His449His and other low frequency *PPARG* polymorphisms. This paper was accepted for publication in the International Journal of Endocrinology in 2014.

Chapter 5: Examines the relationship between indices of IR and carotid intima media thickness (CIMT), a marker of subclinical CVD. The manuscript draft prepared.

Chapter 6: Provides a summary of the findings, an integrated discussion and conclusion as well as the limitations of the study with recommendations.

References: A reference list is included for all the chapters at the end of the dissertation.

Appendices: Listed at the end of the dissertation.

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List of acronyms and abbreviations

AKT/Akt	Protein kinase B
ALARA	As Low As Reasonably Achievable
AS160	Akt substrate of 160kDa
BMI	Body mass index
CAC	Coronary artery calcification
Cbl-CAP complex	Adaptor proteins complex
C3G	Rho-family guanine nucleotide exchange factor
CHD	Coronary heart disease
CIMT	Carotid intima media thickness
cJNK	c-JUN NH ₂ terminal kinase
CPUT	Cape Peninsula University of Technology
CRP	C-reactive protein
CVD	Cardiovascular diseases
DALY	Disability-adjusted life years
DBD	DNA binding domain
4E-BP1	eIF-4E-binding protein
ED	Endothelial dysfunction
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated Glomerular filtration rate
EPIV	Echo particle image velocimetry
ERK	Extracellular signal-regulated kinase
FFA/s	Free fatty acid/s
FIRI	Fasting insulin resistance index
F6P	Fructose-6-phosphate
FTO	Fat mass and obesity associated gene
GCKR	Glucokinase (hexokinase 4) regulator
GFR	Glomerular filtration
GGT	γ -glutamyltransferase
GLUT 4	Glucose transporter 4
GRB2	Growth factor receptor-bound protein 2
GRB14	Growth factor receptor-bound 14
GSK	Glycogen synthase kinase
GWA	Genome-wide association
GWAS	Genome-wide association studies
HbA1c	Glycated hemoglobin A1c

HDL	High-density lipoprotein
HDL-c	High-density lipoprotein cholesterol
HEC	Hyperinsulinaemic euglycaemic clamp
HOMA-IR	Homeostasis model assessment of insulin resistance
HOMA- β %	Homeostasis model assessment of beta cell function
HWE	Hardy Weinberg
IDF	International Diabetes Federation
<i>IGF1</i>	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IKK β	Nuclear factor- κ B transcription factors (IkB α kinase β)
IL-6	Interleukin-6
INSR	Insulin receptor
IR	Insulin resistance
IR/S	Insulin resistance/sensitivity
<i>IRS</i>	Insulin responsive substrate
<i>IRS1</i>	Insulin receptor substrate 1
<i>IRS2</i>	Insulin receptor substrate 2
IR Syndrome/Syndrome X/MetS	Metabolic syndrome
IS	Insulin sensitivity
JNK	c-JUN N-terminal kinase
KLF14	Krüppel-like factor 14
KCNJ11	Potassium inwardly rectifying channel J11
KCNQ1	Potassium voltage-gated channel
LBD	Ligand binding domain
LD	Linkage equilibrium
LDL	Low-density lipoprotein
LDL-c	Low-density lipoprotein cholesterol
MAP	Mitogen-activated protein
MDRD	Modification of diet in renal disease
MetS	Metabolic syndrome
mTOR	Mammalian target of rapamycin
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor -1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDK	Protein kinase 3-phosphoinositide-dependent protein kinase
PH	Pleckstrin homology
PI 3-kinase	Phosphatidylinositol 3-kinase
PIP2 1 & 2	Phosphatidylinositol dependent protein kinases 1 & 2
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PtdIns(3,4,5)P ₃	Phosphorylate phosphatidylinositol biphosphate
PPARE	PPAR response elements
<i>PPARG/PPAR</i> γ	Peroxisome proliferator-activated receptor gamma
PPARs	Peroxisome proliferator-activated receptors
PTB	Phosphotyrosine binding
PAWE	Power of association with errors
QUICKI	Quantitative insulin sensitivity check index
Ras/MAP/MEK	Mitogen-activated protein (MAP) kinase/ MEK kinase
RTPCR	Real-time polymerase chain reaction
RXR	Retinoid X receptor
SAT	Subcutaneous fat
SFA	Saturated fatty acid
SH2	src-homology-2 domain proteins
SHC	Substrate for insulin receptor
S6K	S6 kinase
SMASA	Self-Medication Association of South Africa
SNP/SNPs	Single nucleotide polymorphism/s
SREBP-1c	Sterol regulatory element-binding protein-1c
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TCERG1L	Transcription elongation regulator 1-like
TCF7L2	Transcription factor 7-like 2
TG	Triglycerides
TNF α	Tumour necrosis factor alpha
TyG	Fasting triglycerides and glucose product
VAT	Visceral fat (central/intra-abdominal)
WC	Waist circumference
WHO	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Insulin resistance (IR) is a pathophysiological condition wherein normal insulin levels fail to maintain normal glucose homeostasis. Since the discovery of insulin in 1922 (Banting et al., 1922) this anabolic hormone was shown to play a critical role in the maintenance of glucose homeostasis through the effects of insulin secretion and action to promote glucose transport into muscle and adipose tissue and to inhibit glucose output by the liver. Resistance to these metabolic actions of insulin is typically defined as IR due to the reduced sensitivity to insulin (Reviewed by Wilcox, 2005; Reaven, 2005; Ye, 2007).

In normal glucose homeostasis the pancreas beta cells secrete the glucagon and insulin hormones that by synergistic actions blood glucose levels are regulated. Glucagon is secreted in response to low blood glucose levels and stimulates the liver to breakdown glycogen to release glucose and to convert non-carbohydrate macronutrients into glucose. Insulin is secreted in response to high blood glucose levels and has an opposing effect to glucagon by suppression of hepatic glycogenolysis, stimulation of hepatic glucose uptake for glycogenesis and facilitation of glucose uptake in the insulin-responsive muscle and adipose tissue. In response to reduced blood glucose levels secretion of insulin is decreased to facilitate hepatic glycogenolysis and lipolysis in the adipose tissue. In abnormal glucose homeostasis multiple disturbances may occur namely, hyperglycaemia, impaired insulin secretion, IR, compensatory hyperinsulinaemia with progression to impaired glucose tolerance (IGT) and consequent type 2 diabetes mellitus (T2DM) (Reviewed by DeFronzo, 2004; Szablewski, 2011).

In the development of IGT from normal there is a marked increase in the glucose-stimulated blood insulin levels known as compensatory hyperinsulinaemia. The latter condition results from the inadequate effect of insulin to regulate the blood glucose levels even with normal insulin levels. An inability to maintain an adequate compensatory insulin response results in the progression to IGT which is further associated with severe insulin resistance. Although insulin secretion is increased, beta cell sensitivity to glucose is reduced and tissue sensitivity to insulin is diminished which in concert contributes to the development of type 2 diabetes mellitus (Cefalu, 2001; Bliss, 1993; Himsworth, 1936; DeFronzo, 2004; DeFronzo, 2009; Reaven, 1975).

Himsworth (1936) was the first to recognise diabetes mellitus on the basis of hyperglycaemia occurring both in the presence (insulin resistance) and absence (insulin deficiency) of insulin. As

such IR is a key pathogenic feature of T2DM where both normal and elevated insulin levels produce a diminished biological response commonly referred to as impaired sensitivity to insulin-mediated glucose disposal (DeFronzo, 2009; Cefalu, 2001; Bliss, 1993). Later studies confirmed the role of IR in the pathogenesis of T2DM and showed association with obesity, metabolic syndrome (MetS) and cardiovascular diseases (CVD) (Reviewed by Reaven, 1988; DeFronzo, 1992; Rao, 2001; Reaven, 2005; Ye, 2007).

1.2 INSULIN SIGNALLING PATHWAY (NORMAL)

The signalling mechanism involved in various biological responses to insulin action is initiated by binding of the hormone to the cell membrane and activation of insulin receptor tyrosine kinase. This results in the stimulation of intracellular signalling cascades (White & Kahn, 1994). An overview of the insulin signalling pathway via the insulin receptor substrate (*IRS*)1/phosphatidylinositol 3-kinase (PI 3-kinase) / protein kinase B (Akt) pathway and the Ras / mitogen-activated protein (MAP) kinase pathways are briefly discussed below in Figure 1.1: p 3 (Reviewed by Hooper, 2011).

Under normal physiological conditions, insulin mediates its actions through binding to the insulin receptor (INSR) β subunits, which when phosphorylated activate the tyrosine kinase activity of the insulin responsive substrate (*IRS*) proteins. The tyrosine phosphorylation reactions activate insulin / insulin growth factor (*IGF*) receptors, which in turn, activate the phosphatidylinositol 3-kinase (PI 3-kinase) and extracellular signal-regulated kinase (ERK) cascades, after which the insulin signalling pathways diverge. Phosphorylated *IRS* proteins bind specific src-homology-2 domain proteins (SH2) that proceed with the *IRS*1 / PI 3-kinase / Akt pathway, resulting in autophosphorylation of the INSR and activation that phosphorylates several adaptor/scaffold molecules, including Shc, APS, CAP, c-Cbl and the *IRS* proteins. Tyrosyl phosphorylation of these proteins within specific motifs serves to recruit downstream effectors. Phosphorylation of the insulin receptor substrate (*IRS*)1 or the insulin receptor substrate (*IRS*)2 leads to recruitment of effectors such as the protein phosphatase SHP2, the adaptor protein Nck and the lipid kinase, PI 3-kinase. The *IRS*1 and *IRS*2 binds to SH2 domains in the 55- or 85-kDa regulatory subunits of PI 3-kinase, an important enzyme that promotes: translocation of glucose transporter proteins, glycogen and lipids; protein synthesis; anti-lipolysis and; the control of hepatic gluconeogenesis (Reviewed by Shepherd et al., 1996; White & Kahn, 1994; White, 1998).

The metabolic actions via PI 3-kinases act on serine and threonine kinases such as Akt / protein kinase B/ PKB (Wang et al., 1999), protein kinase C (PKC) (Kotani et al., 1998) and phosphatidylinositol (PI) dependent protein kinases 1 and 2 (PIPD 1&2). The catalytic subunit of PI 3-kinase, p110 phosphorylate PI biphosphate leading to the formation of PtdIns (3, 4, 5) P_3 that is

the downstream effector of Akt/protein kinase B (PKB). Activated Akt enters cytoplasm it leads to phosphorylation and inactivation of glycogen synthase kinase (GSK) 3. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis thus the inactivation of GSK3 by Akt promote glucose storage as glycogen (Reviewed by Hooper, 2011). Activated Akt / protein kinase B also phosphorylates its 160-kDa substrate (AS160), which stimulates the translocation of insulin-mediated glucose transporter (GLUT) 4 from intracellular vesicles to the plasma membrane (Sano et al., 2003). In addition, PI 3-kinase independent pathway provides a second signal to recruit GLUT4 to the plasma membrane through activation of the Cbl-CAP complex. Cbl interacts with the adaptor protein Crk that in turn activates members of the GTP-binding proteins, TC10 that promote GLUT4 translocation (Saltiel & Khan, 2001).

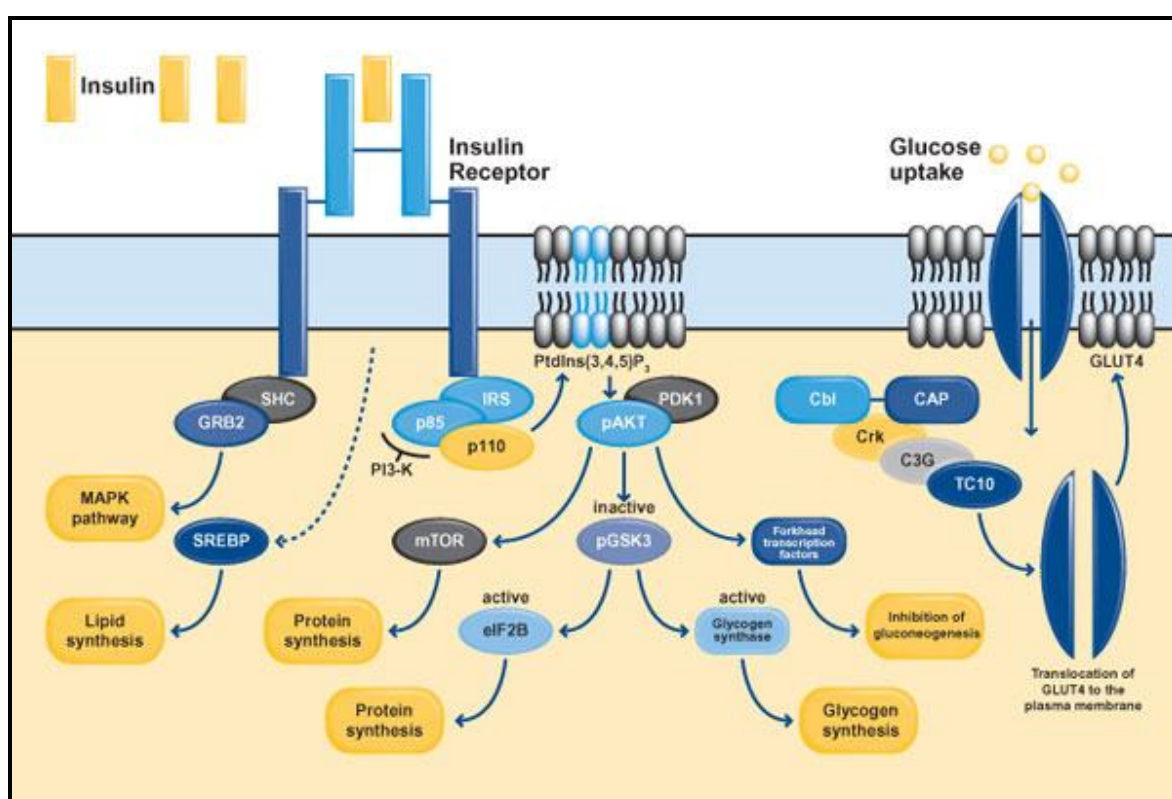


Figure 1.1 An overview of the insulin signalling pathway (Reviewed by Hooper, 2011)

Abbreviations: AKT, protein kinase B; C3G, Rho-family guanine nucleotide exchange factor associated with Cbl-CAP complex; Cbl, Crk, CAP, complex of adaptor proteins; eIF2B, binding protein dephosphorylate to promote protein synthesis; GLUT, glucose transporter; GRB2, growth factor receptor-bound protein 2 an adaptor protein that activate the MAPK cascade; GSK, glycogen synthase kinase; IRS, insulin receptor substrate family; mTOR, mammalian target of rapamycin; MAPK pathway, mitogen-activated protein kinase mitogenic responses independently of IRS; PDK, protein kinase 3-phosphoinositide-dependent protein kinase required for AKT activation; PI 3-K, phosphatidylinositol 3-kinase; p110 and p85, catalytic subunits of PI 3-kinase; PtdIns(3,4,5)P₃, phosphorylate phosphatidylinositol biphosphate; SHC, substrate for the insulin receptor; SREBP, steroid regulatory element-binding protein involved in protein synthesis; TC10, GTP-binding protein family which promote GLUT4 translocation.

The Akt also activates mammalian target of rapamycin (mTOR) an atypical serine/threonine protein kinase that belongs to the PI 3-kinase related kinase family that interacts with several proteins to form mTORC1 and mTORC2. The mTORC1 is activated by nutrients, growth factors and cellular energy status. The substrates of mTORC1 are p70 ribosomal S6 kinase (S6K) and eIF-4E-binding protein (4E-BP1) through which mTOR1 controls protein synthesis. The mTORC2 is activated by association with ribosome in response to growth factors in growing cells. mTORC2 regulates the actin cytoskeleton, cell survival through phosphorylation of Akt and protein kinase C (Dazert & Hall, 2011; Zinzalla et al., 2011).

In the second pathway, the Ras / MAP kinase pathway, Ras proceeds through an adaptor protein that links *IRS1* to the Ras/MAP kinase pathway. Growth factor receptor-bound protein 2 (GRB2), an adaptor protein involved in signal transduction/cell communication, recruits the SOS complex to tyrosyl phosphorylated transforming protein 1 (SHC), which mediates p21ras. This activates the Ras / Raf / MAP kinase (MAPK) / MEK kinase cascade by binding to isoforms ERK1 and ERK2. The Ras pathway activates transcription factors and stimulates growth by promoting the actions of insulin's mitogenic effects (Shulman, 1999; Cusi et al., 2000; Schmitz-Peiffer & Whitehead, 2003).

1.3 PATHOPHYSIOLOGY OF INSULIN RESISTANCE

Insulin resistance is a condition in which cells fail to respond adequately to circulating insulin resulting in high blood glucose levels referred to as impaired insulin-induced glucose uptake. This condition is caused by defects in the insulin signalling pathway, which in turn, increases the secretion of insulin (hyperinsulinaemia) as a compensatory mechanism to maintain normal blood glucose levels associated with type 2 diabetes mellitus (Boden, 2005; Moller & Kaufman, 2005; reviewed by Ye, 2013). In addition, a cluster of cardiovascular disorders that are independent risk factors for CVD have been shown to be strongly associated with insulin resistance. These disorders include dyslipidaemia, obesity, hypertension, glucose intolerance and endothelial dysfunction (ED) and seem to interact to promote the development of diabetes and CVD/atherosclerosis (DeFronzo, 2010; Ikmal et al., 2013). Of these, obesity has been shown to be the most prominent risk factor for the development of IR, where the dysfunction in adipose tissue to insulin action leads to an increase in lipolysis (hyperlipidaemia), resulting in elevated free fatty acids (Wajchenberg, 2000; DeFronzo, 2004; Garg, 2004). Overstimulation of insulin by elevated plasma FFA levels in obese individuals contribute to the increase in plasma insulin levels as a compensatory action and account for up to 50% of IR in obese patients with type 2 diabetes mellitus (Boden, 2001; Mc Garry, 2001; Boden & Shulman, 2002; Ye, 2007; He et al., 2011).

Alternate mechanisms are proposed to be involved in the multistep process of insulin resistance. These include mitochondrial dysfunction (Ozcan et al., 2009; Szendroedi et al., 2012), endoplasmic

reticulum stress (Szendroedi et al., 2012), hypoxia (Hosogai et al., 2007; Ye, 2007; Wood et al., 2009; Prieto et al., 2014) and oxidative stress (Evans et al., 2003; Furukawa et al., 2004; Graham & Adler, 2014). Furthermore, IR is associated with a wide spectrum of metabolic disorders and has been shown to play a crucial role in the pathogenesis of PCOS (Diamanti-Kandarakis & Dunaif, 2012), non-alcoholic fatty liver (Masuoka & Chalasani, 2013) and lipodystrophy (Semple et al., 2011).

1.4 MOLECULAR MECHANISM OF INSULIN RESISTANCE

Several defects in the insulin signalling pathway have been identified, mostly at post-translational level with impaired phosphorylation of the insulin receptors in response to insulin that resulted in insulin resistance. The basis of IR, determined by the insulin signalling cascades, is classified into three categories where the primary effect is either at the pre-receptor level or at the post-receptor level.

1.4.1 The pre-receptor level

The pre-receptor causes of IR involve the production of abnormal insulin and anti-insulin antibodies to the insulin receptors (INSR). Genetic mutations result in the formation of defective insulin, that is: proinsulin is not converted to insulin and thereby affects the binding of insulin to the insulin receptors (Tager et al., 1979; Chan et al., 1987; Steiner et al., 1990).

1.4.2 The post-receptor level

At the post-receptor level, the transmembrane receptor on the cell surface INSR, the defective signal transduction in the *IRS1* / PI 3-kinase / Akt pathway, mutations in the *IRS* proteins, rare mutations in GLUT4 and various serine kinases lead to insulin resistance (Taylor et al., 1990; Ye, 2007; He et al., 2011).

The INSR is activated by insulin, *IGF-I*, *IGF-II* and belongs to the large class of tyrosine kinase receptors (Ward & Lawrence, 2009). The INSR is involved in the post-receptor signalling pathway with a key role in the regulation of glucose homeostasis, which if disrupted, can result in various metabolic states including diabetes (Belfiore et al., 2009). A number of causes related to the development of IR have been identified at the INSR level. A decrease in the number of receptors can lead to failure of tyrosine kinase activity, thereby reducing the binding of insulin. It is suggested that diabetes may be due to a slightly decreased affinity of the receptor for insulin or decreased kinase activity, possibly driven by environmental factors such as obesity (Taira et al., 1989). Relatively rare mutations of the INSR gene have been reported to induce IR and lipodystrophy, among other metabolic disorders (Reviewed by Pankov, 2013). In lipodystrophy, the primary adipose tissue defects induce IR at the post-receptor level by impairing the ability of

insulin to suppress hepatic gluconeogenesis, leading to hyperglycaemia and hyperinsulinaemia (Semple et al., 2011). The excess energy is stored as lipids in ectopic sites, which lead to severe metabolic dysregulation resulting in IR, dyslipidaemia, reproductive and liver complications and other chronic complications related to diabetes and cardiovascular diseases (Vatier et al., 2013).

Studies regarding IR in animal and human models have consistently shown a reduced strength in the insulin signalling via *IRS1* / PI 3-kinase / Akt pathway, resulting in decreased glucose uptake and utilisation in insulin target tissues (Kahn & Flier, 2000; Pessin & Saltiel, 2000). A number of serine kinases have been identified that phosphorylate serine residues of *IRS1* and weaken the insulin signal transduction. This reduces the ability of the IRS protein to attract PI 3-kinase and thereby minimises its activation (Birnbaum, 2001; Aguirre et al., 2002; White, 2003), which can lead to degradation of the *IRS1* protein (Shah et al., 2004). In contrast, excessive serine phosphorylation of *IRS* proteins can be detrimental for normal metabolic insulin signalling downstream and can result in insulin resistance.

Several defects in the insulin signalling pathways in the skeletal muscle of T2DM subjects have been identified, mostly at the level of post-translational regulation of key enzyme activity (Ducruzeau et al., 2001), including impaired phosphorylation of insulin receptors and *IRS1* in response to insulin (Arner et al., 1987; Björnholm et al., 1997; Cusi et al., 2000). The induction of PI 3-kinase, PKC and Akt kinase activity was also reduced (Krook et al., 1998; Cusi et al., 2000; Krook et al., 2000). This induction appears to be critical in the mechanism of insulin action on GLUT4 translocation and glucose transport (Bandyopadhyay et al., 1997; Drazin, 2006). In another study, skeletal muscle of diabetic subjects showed impaired insulin activation of the *IRS1* / PI 3-kinase / Akt signalling pathway, which is a critical step in the regulation of glucose transport in response to insulin (Le Marchand-Brustel et al., 1995; Choi & Kim, 2010). However, the expression of the GLUT4 gene was normal in the muscle of diabetic subjects (Garvey et al., 1988), with the impaired glucose uptake probably due to altered trafficking or the impaired function of glucose transporter 4. The resistance to insulin stimulation was related to impaired insulin signal transduction (Pedersen et al., 1990; Dohm et al., 1991; Kim et al., 1999).

Various other serine kinases are reported to promote serine phosphorylation of *IRS1*, such as c-JUN N-terminal kinases (JNKs), stress-activated protein kinases, PKC and tumour necrosis factor alpha (TNF α). Activation of JNKs by free fatty acids (FFAs), stress and inflammation increase serine phosphorylation of *IRS1*, thereby reducing insulin signalling (Hirosumi et al., 2002). However, when JNKs activation is blocked, the defects induced by FFAs are prevented (Gao et al., 2004). Pro-inflammatory PKC has also been found to cause serine phosphorylation of *IRS1*, with increased PKC and activity of JNKs in the skeletal muscle of obese and T2DM subjects,

demonstrating a potential role in the pathogenesis of insulin resistance (Itani et al., 2001; Bandyopadhyay et al., 2005). The TNF α was shown to be increased in the adipose tissue of obese and IR subjects (Hotamisligil & Spiegelman, 1994; Qi & Pekala, 2000). The TNF α was also found to block insulin signalling by promoting serine phosphorylation of *IRS1*, resulting in a decline in *IRS1* associated PI 3-kinase activity (Hotamisligil et al., 1996; Drazin, 2006).

The mTOR pathway was also found to cause IR as mTOR blocks insulin signalling when stimulated by nutrients and insulin. IR occurs when high mTOR/S6K activity follows over-activation by nutrients, cytokines, insulin and other hormones degrading the *IRS* proteins (Reviewed by Blagosklonny, 2012; Tremblay et al., 2005). The mTOR/S6K kinase signalling pathway was also found to modify insulin-stimulated glucose transport in skeletal muscle cells. Inhibition of insulin-mediated PI 3-kinase activity leads to increased serine/threonine phosphorylation of *IRS1* and decreases binding of the p85 subunit of PI 3-kinase to *IRS1* thereby decreasing cellular glucose uptake (Tremblay et al., 2005, Tremblay et al., 2001). Over-stimulation of beta cells leads to increased insulin secretion, which eventually fails and results in type 2 diabetes mellitus (Blagosklonny, 2012).

Another molecular mechanism that can lead to IR is a disruption in the balance of the number of PI 3-kinase regulatory subunits (Ueki et al., 2002). A balance exists between the regulatory subunits p85 α and p85-p110 heterodimer, with the latter responsible for the PI 3-kinase activity (Shepherd et al., 1998). An imbalance between the subunits could increase or decrease PI 3-kinase activity because both p85 α and p85-p100 compete for the same binding site on the tyrosine-phosphorylated *IRS* proteins. This was evident in studies in which IR states were induced by human placental hormone (Barbour et al., 2004), obesity and diabetes (Bandyopadhyay et al., 2005).

Furthermore, defects at the post-receptor level in the insulin signalling pathway have been linked to a large number of disease associated conditions related to the development of insulin resistance. For example, polycystic ovarian syndrome (PCOS), a metabolic and a reproductive disorder, has been reported to confer a significant increased risk for T2DM associated with IR, independent of obesity (Diamanti-Kandarakis & Dunaif, 2012). The major defect in the insulin action reported was possibly caused by serine phosphorylation of the INSR and *IRS1*, an early stage of insulin transduction present in both adipocytes and skeletal muscle (Dunaif et al., 2001; Ciaraldi et al., 1992; Diamanti-Kandarakis & Dunaif, 2012). Apart from the defects in the insulin signalling pathway, mitochondrial dysfunction was also suggested as a possible contributor to IR in PCOS skeletal muscle (Diamanti-Kandarakis & Dunaif, 2012).

1.5 LIFESTYLE RISK FACTORS

The modifiable lifestyle factors that affect IR include physical activity and diet. Studies show physical activity may reduce body weight, increase FFA oxidation in skeletal muscle and may thereby contribute to a lower risk of IR associated with obesity (Balkau et al., 2008; Malin & Kirwan, 2012; Hellgren et al., 2014). The influence of dietary fat composition on the development of IR in humans show that high-fat diets can lead to changes in adiposity, insulin sensitivity (IS) and mitochondrial dysfunction (Coelho et al., 2011). An excess of calories leads to hyperinsulinaemia, which increases the expression of the SREBP-1c in the beta cells, resulting in lipogenesis and obesity (Unger & Scherer, 2010; Coelho et al., 2011).

The non-modifiable factors identified that affect IR are ethnicity, gender and age. Population studies have shown non-Caucasians are more IR than Caucasians (Svec et al., 1992; Harris, 1990; Haffner et al., 1997; Chandalia et al., 1999; Kasim-Karakas, 2000; Wild et al., 2004). The highest rates of T2DM are found among Native Americans, with a high incidence of IR in the Pima Indians from Arizona in the United States and in natives of the South Pacific islands, such as the Nauru (Knowler et al., 1978; Wild et al., 2004). A high prevalence of T2DM is also seen to be more predominant in Hispanic and African-American populations than in Caucasians and reported to be associated with a higher incidence of insulin resistance (Svec et al., 1992; Kasim-Karakas, 2000; Stefan et al., 2004).

Gender differences in body composition show that men have higher visceral adiposity associated with IR risk factors, such as elevated FFA levels and insulin secretion (Couillard et al., 1999), whilst women have peripheral fat distribution associated with improved IS, compared with central adiposity (Snijder et al., 2003). Oestrogen has a favourable effect on insulin and glucose homeostasis (Louet et al., 2004), adipose tissue distribution and pro-inflammatory markers (Dantas & Sandberg, 2005). However, lack of oestrogen synthesis in men is associated with insulin resistance (Smith et al., 1995). A high prevalence of IR in ageing people and was found to contribute to increased risk of diabetes, mainly due to increased visceral adiposity, imbalance of sex hormones and lack of physical activity (Gabriely et al., 2002; Sakurai et al., 2010).

1.6 ESTIMATION OF INSULIN RESISTANCE/SENSITIVITY

The gold standard for the estimation of IR is the hyperinsulinaemic euglycaemic clamp (HEC) technique, a reference method for the direct quantification of insulin sensitivity. The alternative method is the minimal model analysis of frequently sampled intravenous glucose tolerance (DeFronzo et al., 1979; Bergman et al., 1979; Muniyappa et al., 2008). In the HEC technique, insulin is infused to achieve steady-state conditions for plasma insulin, blood glucose and the glucose infusion rate (DeFronzo et al., 1979). The several rates of insulin infusion allow the

establishment of a relationship between the whole body glucose disposal and plasma insulin levels. This enables the discrimination between the states of decreased IR and/or the altered maximal capacity to dispose of glucose (Robert, 1995). The glucose measurements taken under clamp conditions are used to assess whether hepatic glucose is suppressed, thus enabling the differentiation between hepatic and peripheral insulin resistance. However, this technique has been found to be cumbersome and only applicable to a small number of subjects within research centres, which limits its usefulness in large epidemiology studies (Muniyappa et al., 2008).

There are several non-invasive alternatives to the HEC technique, among which the oral glucose tolerance test (OGTT) has been used to diagnose glucose tolerance and type 2 diabetes mellitus. The OGTT reflects the efficiency of the body to dispose of glucose after an oral glucose load or meal, with multiple measures of glucose and insulin being obtained at different intervals. Several surrogate markers are derived from this indirect quantification method for insulin sensitivity (Muniyappa et al., 2008; Borai et al., 2011). These surrogate markers are based on dynamic tests, taking into account both the fasting steady-state conditions and the postload glucose and insulin levels. The estimation of IS with OGTT correlates well with the clamp technique (Matsuda & DeFronzo, 1999; Hanley et al., 2003).

Currently, simpler indices such as the homeostasis model assessment insulin resistance (HOMA-IR), homeostasis model assessment of functional beta-cells (HOMA- $\beta\%$) (Matthews et al., 1985) and the quantitative insulin sensitivity check index (QUICKI) (Katz et al., 2000) are used in most studies for IR assessment (Borai et al., 2009; Antuna-Puente et al., 2011). These OGTT-derived indices mimic the physiological response of the postload glucose-insulin interaction and are estimated from a fasting blood sample that is taken following an oral glucose load (Borai et al., 2011). The HOMA-IR index is regarded as a simple, inexpensive and reliable surrogate measure of IR that has been validated against the HEC technique and found to reflect more hepatic than peripheral IR, whereas the HOMA- $\beta\%$ is a much less robust estimate of beta cell function (Wallace et al., 2004; Boyko & Jensen, 2007; McAuley et al., 2007; Buchanan et al., 2010). The QUICKI index is very similar to HOMA-IR but is based on the logarithms and reciprocal of the fasting glucose-insulin product. Results of this index showed greater accuracy than HOMA-IR over a broad range of IS, and it was found to have a stronger correlation with the HEC technique (Katz et al., 2000; Yokoyama et al., 2004). These indices have since been upgraded and modified by including additional parameters into the formulae. For example, the substitution of the fasting C-peptide for insulin in the HOMA-IR formula can be used for subjects with diabetes that are insulin treated (Li et al., 2004). The addition of a fasting non-esterified fatty acid concentration into the QUICKI formula has been reported to improve the assessment of IR through better correlation with the clamp-based index of insulin sensitivity (Ijzerman et al., 2009).

Several population studies have since validated these simple, inexpensive surrogate markers and highlighted important limitations that should be taken into account when they are used in large epidemiologic studies (Borai et al., 2007; Borai et al., 2011). Furthermore, studies in biochemical markers with insulin regulated proteins found them to be more reliable markers of IR than fasting insulin levels (Birkeland et al., 1993; Maddux et al., 2006; Borai et al., 2011). For example, the ratios of biochemical markers such as leptin/adiponectin and triglycerides (TG) / high density lipoprotein cholesterol (HDL-c) are shown to be useful markers of insulin resistance (Oda et al., 2008; Roa et al., 2009; Finucane et al., 2009). To ensure reliability, it is suggested that a combination of biomarkers and other parameters, such as the anthropometric measurements of age, gender and ethnicity, be used to estimate insulin resistance (Borai et al., 2011).

1.7 DISEASE CONDITIONS ASSOCIATED WITH INSULIN RESISTANCE AND CARDIOVASCULAR DISEASE

Over the years, several studies have identified obesity as a major risk factor for the development of IR, T2DM, MetS and CVD, thereby providing evidence that these conditions may all be interwoven.

1.7.1 Obesity induced insulin resistance

Obesity-associated risk factors that may induce IR include the following: (1) hyperinsulinaemia and FFAs; (2) inflammation; and (3) fat distribution.

1.7.1.1 Hyperinsulinaemia and free fatty acids

Defects in the insulin signalling pathway cause IR, which in turn, increases secretion of insulin (hyperinsulinaemia) (Boden, 2005; Moller & Kaufman, 2005; Reviewed by Ye, 2013). The dysfunction in adipose tissue of insulin action leads to an increase in lipolysis (hyperlipidaemia), resulting in elevated free fatty acids (Wajchenberg, 2000; Garg, 2004; DeFronzo, 2004). Higher lipolysis activity in visceral adiposity seems to be primarily responsible for the excessive IR-related FFA flux to the liver and skeletal muscle (DeFronzo, 2004; Kovacs & Stumvoll, 2005). In the muscle, increased FFAs are found to promote fatty acid oxidation, leading to accumulated glucose-6-phosphate and intramyocellular triglyceride storage, with a decrease in glucose uptake (Randle et al., 1963).

Further studies concerning the mechanism by which increased FFAs induce IR in muscle showed that this inhibition of glucose transport activity seems to be linked to the decreased *IRS1* associated with PI 3-kinase activity (Cline et al., 1999; Dresner et al., 1999; Shulman, 1999). The PI 3-kinase is an important enzyme involved in the translocation of glucose transporter proteins, glycogen, lipid and protein synthesis, as well as anti-lipolysis and control of hepatic gluconeogenesis (Wilden

et al., 1992; Shepherd et al., 1996; White, 1998; Rhodes & White, 2002). Furthermore, FFAs are involved with hepatic autoregulation in the liver, in which FFAs stimulate gluconeogenesis but not hepatic glucose production. However, defects in hepatic autoregulation due to increased FFA flux leads to increased hepatic glucose production, which results in the decrease of glycogen storage in the liver (Boden, 2001; Lam et al., 2003). In addition, insulin secretion is altered in response to the increased FFAs, and this obstructs insulin suppression of glycogenolysis, thereby interfering with the glucose-stimulated insulin secretion (Boden, 2001; Lam et al., 2003; Delarue & Magnan, 2007).

Free fatty acids are also known to cause defects in insulin signalling through interference via protein kinase C-induced serine phosphorylation of *IRS1* (Yu et al., 2002). This causes significant functional alterations that could have adverse effects on glucose transport (Pessin & Saltiel, 2000; Chavez et al., 2003; Bhattacharya et al., 2007). Evidence indicates that FFAs may be involved in diabetes and obesity by mediating insulin resistance (Randle et al., 1963; Boden, 1997; Shulman, 1999; Boden & Shulman, 2002; Kovacs & Stumvoll, 2005; Delarue & Magnan, 2007).

1.7.1.2 Inflammation

During the inflammatory response in adipose tissue, adipocytes increase the secretion of pro-inflammatory cytokines, causing systemic inflammation (Reviewed by Coppack, 2001; Ye, 2013). Adiponectin, TNF α and leptin have been associated with obesity and insulin resistance. It is suggested that of these, adiponectin is a stronger marker for both IR and the risk of T2DM by contributing to both IS and glucose homeostasis (Lawlor et al., 2005). Significantly higher adiponectin levels were observed in females, possibly due to the inhibitory effect of androgens on adiponectin (Nishizawa et al., 2002), higher visceral adiposity in females (Couillard et al., 1999) or lower IS found in males (Geer & Shen, 2009). These differences may contribute to a more insulin-sensitive environment in females than in males (Geer & Shen, 2009). Furthermore, genetically determined levels of adiponectin were also found to influence IS in the development of IR and diabetes, independent of adiposity (Gao et al., 2013).

TNF α was the first cytokine associated with the pathogenesis of obesity and insulin resistance. The increased expression of TNF α was positively correlated with adiposity and insulin resistance (Hotamisligil et al., 1993; Hotamisligil & Spiegelman, 1994; Ryden et al., 2002; Ruan & Londish, 2003). The TNF α produced by the macrophages that infiltrate adipose tissue is highlighted as a major link between inflammation and insulin resistance (Sanshiro et al., 2013). This cytokine activates pro-inflammatory signal cascades that inhibit the INSR signalling through serine phosphorylation of *IRS1*, thereby reducing the GLUT4 gene expression (Weisberg et al., 2003).

Other cytokines associated with obesity-induced IR and inflammation include interleukin-6 (IL-6) (Kim et al., 2009), plasminogen activator inhibitor-1 (PAI-1) (Alessi et al., 2007), retinol-binding protein 4 (RBP4) (Yang et al., 2005) and resistin (Bajaj et al., 2004).

Leptin was found to be significantly higher in obese women and strongly correlated with IR, despite adjusting for body mass index (BMI) and gender (Silha et al., 2003). Higher leptin concentration was also reported in females compared with males, associated with larger adipocytes and with subcutaneous fat (Garaulet et al., 2000). Gender differences in the concentration of leptin may be due to oestrogen (Garaulet et al., 2000). An increase in body fat mass has also been linked to leptin and IR (Cnop et al., 2002), but others reported an independent relationship with body fat mass (Appleton et al., 2002; Fischer et al., 2002).

Inflammation was also found to inhibit insulin signalling activity in adipocytes and hepatocytes, while the insulin action in muscle was unaffected (White, 2002; Ye, 2011). Several mechanisms involving two pathways were proposed to explain this interference in insulin signalling, where signalling molecules in the inflammatory pathways such as I κ B α kinase β (IKK β) and JNK 1 were found to be activated in adipose tissue and the liver. The serine kinase isoform IKK β was found to inhibit insulin signalling by phosphorylation of *IRS1* at multiple serine residues in adipocytes (Gao et al., 2002), and the activity of JNK involved in the pathogenesis of IR by phosphorylation of *IRS1* in humans inhibited the signalling pathway in response to tumour necrosis factor alpha (Aguirre et al., 2000).

1.7.1.3 The role of fat distribution

Over the last 25 years, a large number of studies investigated the association of body fat distribution and metabolism in obesity with IR and diabetes (Tchernof & Després, 2013). Adiposity measurements are usually defined in terms of BMI, waist circumference (WC) and the region of fat distribution in terms of visceral adiposity (VAT) or central / intra-abdominal and subcutaneous adiposity (SAT) (Kahn & Flier, 2000). Both VAT and SAT release elevated FFAs into the circulation with adverse effects, which include the inhibition of insulin-stimulated glucose uptake, glycogen synthesis and glucose oxidation (Boden, 2001). The VAT is metabolically more active than SAT with regard to the increased FFA flux. The increased FFAs promote IR at a cellular level by an increased production of hepatic very low-density lipoprotein (Giorgino et al., 2005).

Furthermore, VAT is linked with ED and the production of significantly higher levels of proinflammatory cytokines compared with subcutaneous adiposity. These pro-inflammatory cytokines include C-reactive protein (CRP), IL-6 and PAI-1, which could possibly explain the stronger association of VAT with insulin resistance (Hashimoto et al., 1998; Lemieux et al., 2001;

Kershaw & Flier, 2004; Preis et al., 2010). Differences in the cytokine secretion profiles were also reported between the SAT and visceral adiposity. The SAT was found to release increased leptin levels, whereas VAT mainly releases TNF α , which in turn influenced the secretion of adiponectin (Ouchi et al., 2003; Reviewed by Fain, 2010). The inverse association between VAT and adiponectin levels was reported to be of higher significance than the association between SAT and adiponectin (Kershaw & Flier, 2004; Kwon et al., 2005; Lawlor et al., 2005). Low levels of adiponectin, higher leptin levels and low-grade inflammation generally observed in obesity have been associated with IR and metabolic syndrome (Matsuzawa et al., 2004).

1.7.2 Insulin resistance, type 2 diabetes mellitus, metabolic syndrome and cardiovascular disease

1.7.2.1 Type 2 diabetes mellitus

An abundance of pathways leads to IR, in which the combination of IR and hyperinsulinaemia provoke multiple associated diseases. Among these, the progression of T2DM, MetS and CVD have shown significant adverse health effects. The association of obesity and T2DM has been well studied, with obesity being the major promoter of insulin resistance (Kahn & Flier, 2000). The association between obesity and IR is a key feature of most individuals with T2DM, which results in the reduced response of the insulin-sensitive tissue to insulin; thereby contributing to the lower IS (Hossain et al., 2007). Thus, the obesity-induced IR fuels the development of T2DM, in which beta cell dysfunction and inadequate insulin secretion cause elevated fasting blood glucose levels (hyperglycaemia), possibly resulting in glucotoxicity (Garvey et al., 1985; Kahn et al., 2006). Insulin resistance is established as a strong link between T2DM and CVD. Insulin resistance accompanied with ED in T2DM patients, worsens the state of subclinical CVD/atherosclerosis, which is a major cause of mortality in diabetic individuals (Ikmal et al., 2013). Furthermore, with the increased obesity and MetS prevalence rates, CVD mortality and morbidity are expected to increase further, posing a greater burden on public health care systems (Go As et al., 2013).

1.7.2.2 Metabolic syndrome

Metabolic syndrome comprises a cluster of risk factors of metabolic origin, including: hyperglycaemia, obesity, dyslipidaemia and hypertension. Insulin resistance has been identified as a key factor in the development of the obesity, dyslipidaemia and hypertension associated with metabolic syndrome (Reaven, 1995, Reaven, 2012, Metabolic Syndrome Clinical Key, 2013). The atherogenic lipoprotein profile of IR/hyperinsulinaemia includes the presence of smaller, denser very low density lipoproteins that initiate lipoprotein changes resulting in increased levels of remnant particles, small low density lipoproteins and low HDL-c, all defined as risk factors for cardiovascular diseases (Reaven, 2005, Reaven 2012). Although not all cases of IR develop

T2DM, most share some of the cluster of characteristics of MetS and this increases their risk of cardiovascular diseases (Reaven, 2012). Obesity, especially central adiposity, promotes IR that mediates all the metabolic disturbances associated with MetS and insulin resistance. Cardiovascular disease morbidity in the obese and overweight T2DM subjects has often been ascribed to metabolic syndrome (Reaven, 2005; Després et al., 2008; Metabolic Syndrome Clinical Key, 2013). This is supported by evidence that showed MetS to confer a 5-fold increased risk of T2DM and a 2-fold increased risk of CVD over a five to ten year period (Alberti et al., 2009).

1.7.2.3 Cardiovascular disease

Insulin resistance and CVD share many characteristics, including dyslipidaemia, hypertension and hyperglycaemia. Insulin resistance promotes the development of a highly atherogenic lipid profile with high TG and low HDL-c and is associated with both diabetes mellitus and cardiovascular diseases (Pansuria et al., 2012). The changing lipid profile associated with IR, referred to as dyslipidaemia, is initiated by increased FFAs due to the defective uptake by adipocytes and leads to increased hydrolysis of triglycerides (Ginsberg, 2000; Boden & Shulman, 2002). Furthermore, IR facilitates the development of hypertension via increased reabsorption of sodium and water by kidney tubule cells, resulting in volume-retention with increased sympathetic stimulation and renin-angiotensin activity (Berne et al., 1992; Galletti et al., 1997; Furuhashi et al., 2004; Lamounier-Zepter et al., 2006). Hypertension is strongly associated with two major risk factors for CVD, namely arterial stiffness and left ventricular dysfunction referred to as vascular ED, and may lead to atherogenesis (Vlachopoulos & O'Rourke, 2000; Hsueh & Quiñones, 2003; Crichton et al., 2014).

Lastly, IR leads to hyperglycaemia in which changes in the glycaemic status can alter lipoprotein and lipid flux that in turn can modulate IS and glucose disposal. Hyperglycaemia may also directly affect inflammation, IS, altered carbohydrates and lipid metabolism in the liver, which may add to vessel injury (Mazzone et al., 2008). Multiple risk factors that contribute to the development of IR, e.g. dyslipidaemia and hyperglycaemia, have also been found to contribute to endothelial dysfunction. The progression of IR to T2DM is similar to the progression of ED to atherosclerosis (Reviewed by Pansuria et al., 2012; Ikmal et al., 2013).

Over the years it has become clear that IR and ED play a central role in the pathogenesis of atherosclerosis. Atherosclerosis is a disease of arterial lipid deposition characterised by a defined series of changes in the vessel wall during atherogenesis referred to as endothelial dysfunction (Radar & Daugherty, 2008). During early onset of the atherogenic process changes in the endothelial cell phenotype impairs the release of nitric oxide (NO), which has multiple vascular protective actions (Hsueh et al., 2003). This occurs through reduced biosynthesis of NO and

increased degradation of oxidative stress. In addition, insulin has also been shown to be involved in vascular actions through stimulation of the production of NO by the endothelium, leading to vasodilation for increased blood flow and glucose disposal in skeletal muscle (Baron & Clark, 1997; Vincent et al., 2004).

During the early stages of atherosclerosis impaired insulin signalling also occurs within various pathways in the macrophages and endothelial and vascular smooth cells (Bornfeldt & Tabas, 2011). Altered insulin signalling in endothelial cells results in the development of ED, which has been highlighted as an important mechanism contributing to progressive atherosclerosis, along with the pro-inflammatory state induced by IR (Aziz & Wheatcroft, 2011). Furthermore, in IR the alteration in insulin signalling PI 3-kinase pathways result in imbalances between NO production and secretion of vasoconstrictor endothelin-1 (ET-1), both of which contribute to a pro-atherogenic effect (Muniyappa, 2013, Ikmal et al., 2013). The inhibition of the anti-atherogenic PI 3-kinase mediated insulin receptor-signalling pathway and over activity of the pro-atherogenic MAP kinase pathway in IR states lead to accelerated atherosclerosis (Bansilal et al., 2007). Insulin resistance is also characterised by hyperinsulinaemia; increased FFA, TG and LDL and decreased HDL-c levels; NO deficiency; and release of various pro-inflammatory cytokines leading to inflammation. All of the latter further worsen ED and contribute to atherosclerosis (Crosio & DeFronzo, 2006; Ikmal et al., 2013; Muniyappa, 2013).

1.8 ASSESSMENT OF SUBCLINICAL ATHEROSCLEROSIS

Nitric oxide deficiency result in ED which is one of the early events in atherogenesis referred to as subclinical atherosclerosis and precedes the development of plaques resulting in the stiffening, hardening and eventual occlusion of the arteries (Celermajer et al., 1992; Balasubramanian et al., 2012; Ikmal et al., 2013; Muniyappa, 2013). The different stages of atherosclerosis have been assessed using several non-invasive methods. The most common of these is B-mode ultrasound used to assess brachial artery flow-mediated dilation (FMD), carotid intima media thickness (CIMT) and carotid plaques.

The FMD measurement evaluates the function of the endothelium in the brachial artery. The endothelium controls vascular function and structure by the production and release of nitric oxide. This technique measures the ability of the arteries to respond with endothelial NO release during reactive hyperaemia (flow-mediated) after a 5-minute occlusion of the brachial artery with a blood pressure cuff. Abnormal values represent ED (Celermajer et al., 1992; Patti et al., 2005). Impaired FMD is predisposing to atherosclerosis and CVD and is a significant predictor of target organ damage after adjustment for several known risk factors (Patti et al., 2005; Yang et al., 2014).

The CIMT refers to the structural anatomical thickness of the arterial wall and is a valuable marker in screening for subclinical atherosclerosis (Pignoli et al., 1986). This method provides a graded measure of vascular damage obtained by measurements of the common carotid arteries with a B-mode image composed of two parallel echogenic lines separated by a hypoechoic space. The distance between the two lines, i.e. the B-mode image of the intimal and medial thicknesses, are measured and then correlated with the thickness of different combinations of tunicae evaluated by gross and microscopic examination (Pignoli et al., 1986; Bertoni et al., 2007). Increased CIMT is associated with CVD and is an independent predictor of future clinical events such as myocardial infarction and stroke (Cobble & Bale, 2010). Large clinical studies have also shown that CIMT predicts CVD risk which includes the Kuopio Ischaemic Heart Disease Study (Salonen & Salonen, 1991), the Cardiovascular Health Study (O'Leary et al., 1992), the Rotterdam Study (Bots et al., 1997), the Atherosclerosis Risk in Communities Study (Chambless et al., 1997), the Malmo Diet and Cancer Study (Rosvall et al., 2005) and the Carotid Atherosclerosis Progression Study (Lorenz et al., 2010).

In the advanced stages of atherosclerosis appearance of carotid plaques and progression to calcification leads to arterial wall injury. The carotid plaques are focal lesions containing lipids and fibrous tissue in the arterial wall that either partially or completely obstructs the lumen, particularly in areas with a severely thickened arterial wall. The presence of calcium in the vessel wall is measured by the coronary artery calcification (CAC) score that is determined by electron beam or multi-slice computed tomography (Agatston et al., 1990; Frink et al., 1970). In a systematic review by Peters et al. (2012) it was concluded that CAC scoring improved stratification of CVD risk categories when added to traditional risk factors (Peters et al., 2012).

In addition to these techniques two newer tests have been developed namely, echo particle image velocimetry (EPIV) and magnetic resonance imaging (MRI) velocimetry. The EPIV technique is used in measurements of shear wall stress through vector analysis of the blood velocities on the wall at the carotid bifurcation. Change in the shear wall stress is considered a causative factor in atherosclerosis and possible rupture of focal lesions (Taxon, 1995; Shaaban & Duerinckx, 2000). EPIV analysis a range of parameters namely: vascular speed profile, velocity vector, speeds maps and haemodynamic blood flow to the wall shear stress (DeMarchi & White, 2012; Castellon & Bogdanova, 2013). Magnetic resonance imaging velocimetry on the other hand assesses changes in the carotid wall thickness by parameters of the velocity profile, shear rate and the general flow stress of the wall. The MRI velocimetry technique is more sensitive and reliable than echo particle image velocimetry (Castellon & Bogdanova, 2013).

1.9 EPIDEMIOLOGY OF INSULIN RESISTANCE AND TYPE 2 DIABETES MELLITUS

Insulin resistance is a common pathophysiological trait that covers a broad clinical spectrum, in which the decreased ability of insulin to perform its biological functions represent the primary physiological defect underlying a constellation of traits, commonly referred to as IR Syndrome (IRS)/Syndrome X /MetS. These traits include IR/hyperinsulinaemia, glucose intolerance, visceral obesity, dyslipidaemia, hypertension and increased prothrombic and anti-fibrinolytic states (National Cholesterol Education Program, 2002). There is no known global prevalence rate for insulin resistance.

However, an overview of the worldwide and South African prevalence rates of obesity and T2DM are briefly discussed because of the pivotal role IR plays in the development of these metabolic states. The IR status in humans is associated with the global increase in obesity, which is driven by environmental changes in diet and physical activity, including a substantial genetic influence leading to the development of common metabolic disorders such as T2DM and cardiovascular diseases (World Health Organization (WHO), 2004).

1.9.1 Global prevalence of obesity and type 2 diabetes mellitus

Obesity is currently emerging as a global epidemic, affecting more than 200 million men and almost 300 million women worldwide, accounting for 10% of the adult population (WHO, 2013). In developing countries, the epidemic of obesity over the last few decades has been the driving force leading to an increased prevalence of IR, MetS and T2DM that is predicted to increase the risk of developing atherosclerosis. In 2010, it was estimated that worldwide, 285 million (6,4%) individuals between the ages of 20 to 79 years had been diagnosed with T2DM, with an expected rise to 552 million (9,9%) diabetics by 2030 (Shaw et al., 2010; Whiting et al., 2011). According to the International Diabetes Federation (IDF), estimates for 2013 showed a substantial increase from their 2010 predictions, with 382 million adults (8,3%) reportedly diagnosed with diabetes. The majority of these individuals were aged between 40 and 59 years, and 80% of them lived in low- and middle-income countries. However, the burden of disease is not only measured by the number of people with diabetes but is also based on the increasing number of deaths caused by the disease. In 2013, about half the deaths in adults were related to diabetes, with most under the age of 60 years, whilst in developing countries such as Sub-Saharan Africa, the proportion was reported to be as high as 75% (IDF, 2013).

In the African region, it is estimated that 19,8 million people have diabetes, of which at least 64% are undiagnosed cases. Diabetes-related deaths for 2013 have been reported to be as high as 522 600 (IDF, 2013). This rapid increase in diabetes is linked to an increase in lifestyle-related, non-communicable diseases, especially the rising obesity prevalence in urbanised African populations

(IDF, 2013). Important changes in lifestyle as a result of the rapid urbanisation of African populations include smoking, drinking, urban poverty and dietary changes, in which the traditional high-carbohydrate / low-fat diet has been replaced by a high-fat / high-carbohydrate Western diet (MacIntyre et al., 2002). In Sub-Saharan African populations, obesity has also been identified as a major risk factor that predisposes to diabetes (Wilkinson et al., 2005). The increase in the prevalence of T2DM is closely linked to the rise in obesity, with about 90% of diabetes cases being attributed to excess weight (Bos & Agyemang, 2013; MacIntyre et al., 2002; Voster, 2002). Insulin resistance-associated diseases are increasing worldwide, with developing countries in Africa following a similar trend but with variations reported between ethnic populations, mainly due to genetic and environmental differences (Reimann et al., 2007).

1.9.2 South African prevalence of obesity and type 2 diabetes mellitus

The highest rate of obesity among adults in Sub-Saharan Africa is found in South Africa. Reports for 1998 showed the overall prevalence of overweight and obesity to be 29% in males and 56% in females (Goedecke et al., 1998), while current reports show a substantial increase in obesity, with rates of 39% reported in males and 69,3% in females (Ng et al., 2014). Overall prevalence rates of overweight and obesity according to ethnicity are available from an earlier study (Puoane et al., 2002), while more recent data for ethnic groups are available for obesity only. Puoane et al. (2002) demonstrated the highest prevalence of overweight and obesity in black females (58,5%), followed by females of mixed-ancestry (52%), white females (49,2%) and Indian females (48,9%). According to a recent survey by the South African Medical Research Council, 33% of black females and 25% of coloured, white and Indian females over the age of 35 years were reported to be obese (overweight not included) In contrast, the survey reported the highest prevalence of obesity (overweight not included) in white males (18%), followed by Indian males (9%), coloured males (8%) and black males (6%) (Self-Medication Association of South Africa (SMASA), 2013).

Global estimates of the incidence of diabetes are based on risk factors, in which obesity in particular is identified as a major risk factor (Barnes, 2011). In South Africa, rapid changes in the structure of diet, physical activities and obesity patterns in urban areas have had a great impact on the prevalence of disease risk related to obesity and diabetes (Reviewed by Vorster et al., 2002). In 2000, diabetes was ranked among the top ten leading causes of death in South Africa, with a prevalence rate of 5,5% for individuals over 30 years old, accounting for 4,3% of deaths and a total of 258 000 disability-adjusted life years (DALY). One DALY represents the loss of the equivalent of one year of full health (Bradshaw et al., 2007). Recent reports from the IDF Diabetes Atlas (2013) showed an increase in prevalence to 8,3% (Guariguata et al., 2014).

The prevalence of T2DM in the South African mixed-ancestry (coloured) population has increased from 10,8% in 1999 (Levitt et al., 1999) to 26,3% in 2012 (Erasmus et al., 2012). Early epidemiological studies investigating diabetes in black South Africans reported lower prevalence rates for this group than for the mixed-ancestry population, with rates varying between 0,02% to 1,3% (Seftel & Abrahams, 1960; Jackson, 1972). However, later studies using the WHO 1985 criteria showed an increased prevalence rate for black South Africans ranging from 4% to 8% (Levitt et al., 1993; Omar et al., 1993; Mollentze et al., 1995). More recent studies reported prevalence rates according to urban and rural areas. In a study by Erasmus et al. (2001) the prevalence of diabetes was 4,5 % with the highest incidence of diabetes observed in the age group from 40-59 years in peri-urban Xhosa speaking factory workers from Transkei, South Africa. Motala et al. (2008) reported 3,9% overall prevalence of diabetes in rural black Africans, 3,5% in males and 3,9% in females, with a marked increase (8,8%, not age adjusted) in the age group 55 to 65 years (Motala et al., 2008). Peer et al. (2012) reported an increased prevalence (13,1%) and also a higher peak age (65 to 74 years) in black Africans in an urban setting. The high rate of diabetes was attributed to the raised adiposity that was present in $\geq 50\%$ of the total study sample and in over 80% of the participants with diabetes (Peer et al., 2012). Following the trend of Sub-Saharan African countries, the burden of diabetes is expected to increase even further due to the high rates of IGT and the extremely high rates of obesity observed in the black and mixed-ancestry populations in South Africa.

Evidence from earlier population based epidemiological studies reported a relatively high occurrence of IR and T2DM in black South African women (Levitt et al., 1993; Van der Merwe et al., 2000). Van der Merwe et al. (2000) demonstrated a higher degree of IR in black, obese females than in white, obese females, even though black females had less VAT. Similar SAT levels were found in both groups (sample size $N = 20$). However, the centralisation of fat still conferred risk (Jennings et al., 2009). Jennings et al. (2009) demonstrated that VAT, as measured by WC, was a better predictor of HOMA-IR than the IDF or ATP III metabolic syndrome criteria.

1.10 GENETICS OF INSULIN RESISTANCE

Insulin resistance and pancreatic beta cell dysfunction are the two major diabetes-related phenotypes influenced by genetic and environmental factors. However, most of the T2DM susceptibility loci are linked to insulin secretion rather than IR (Imamura & Maeda, 2011). The GWAS carried out primarily in individuals of European descent have been identified about 40 susceptibility loci for T2DM by 2010 (Dupuis et al., 2010; Voight et al., 2010; Brunetti et al., 2014). The numbers of new loci being discovered are continuously increasing with genetic studies currently identifying more than 65 genetic variants that increase the risk of T2DM by 10 - 30% (Ayub et al., 2014; Morris et al., 2012). Of these the majority are directly linked to pancreatic beta

cell dysfunction, with only a few directly mapped to insulin resistance (Scott et al., 2007; Zeggini & McCarthy, 2007; Sladek et al., 2007; Zeggini et al., 2008; Imamura & Maeda, 2011). The paucity of IR genes found through GWAS could possibly be explained by several reasons. The lower heritability of IR traits that indicates a stronger influence of environmental covariates, ascertainment criteria may impose constraints around the IR measures that result in variance, the measures of IS in large epidemiology studies may be poorly correlated with IR at tissue or molecular level, variants that affect IR may be less frequent and have more modest effect sizes (Florez et al., 2008).

In addition, T2DM risk alleles at three loci, namely peroxisome proliferator-activated receptor (*PPARG*), fat mass and obesity-associated (*FTO*) and Krüppel-like factor 14 (*KLF14*) genes have also been associated with higher fasting insulin and reduced IS (Voight et al., 2010). There also appears to be an overlap with the obesity, IR and T2DM loci. For example, the *FTO* gene (Scuteri et al., 2007) was first identified as a T2DM susceptibility locus (Scott et al., 2007; Zeggini & McCarthy, 2007), with other studies that showed a strong association between the *FTO* variants and T2DM, mostly mediated by an increase in body weight with varying degrees of association with insulin resistance (Frayling et al., 2007; Omori et al., 2008).

Recently the importance of T2DM loci, well known in other populations, has been investigated in Africans. Of these loci the role of candidate genes transcription factor 7-like 2 (*TCF7L2*), *PPARG*, potassium inwardly-rectifying channel J11 (*KCNJ11*), *CAPN10* and several other GWA genes namely, potassium voltage-gated channel (*KCNQ1*), *MLXIPL*, *SLC308*, *ENNP1* and *IGF2BP2* associations with T2DM and phenotypic traits were examined (Danquah et al., 2013; Turki et al., 2012; Turki et al., 2013; Mtiraoui et al., 2012). The *TCF7L2* gene is involved in insulin secretion and the most strongly associated with T2DM in Caucasians (Dupuis et al., 2010) as well as in African Americans (Saxena et al., 2012), which was also confirmed in Ghanaians (Danquah et al., 2013), Nigerians (Helgason et al., 2007) and Tunisians (Turki et al., 2013; Mtiraoui et al., 2012). However, the protective allele against T2DM of the *PPARG* gene (Saxena et al., 2012) was rare in Ghanaians but absent in black South Africans from Zulu descent (Danquah et al., 2013; Pirie et al., 2010). Similarly the risk-conferring alleles of *KCNJ11* and *CAPN10*, which are both related to insulin secretion, were not significantly associated with T2DM in Ghanaians or black South Africans (Danquah et al., 2013; Pirie et al., 2010). Furthermore, in Tunisians no association with T2DM was found with *KCNQ1*, *ENNP1*, *MLXIPL*, *IGF2BP2* and *PPARG* but *KCNJ11* and *SLC308* were associated with type 2 diabetes mellitus (Turki et al., 2012; Mtiraoui et al., 2012).

The interest in this study is the IR loci of which the *PPARG*, *IRS1*, glucokinase (*GCK*), glucokinase (hexokinase 4) regulator (*GCKR*) and insulin-like growth factor 1 (*IGF1*) have already

been identified (Watanabe, 2010; Povel et al., 2012). In addition, a few other genes were also found to be associated with both T2DM and IR, namely the INSR, growth factor receptor-bound 14 (GRB14), transcription elongation regulator 1-like (TCERG1L) and sterol regulatory element-binding transcription factor 1 (SC4MOL) (Brunetti et al., 2014). These IR loci are briefly summarised below in Table 1.1: p 21.

1.10.1 Common variants in the *PPARG* gene associated with insulin resistance

The *PPARG* gene is a member of the super family of nuclear receptors involved in adipocyte differentiation (Evans et al., 2004). The *PPARG* form heterodimers with retinoid X receptors (RXRs) that bind to a complex of oligonucleotides containing peroxisome proliferator response elements and together with functional co-activators lead to an increase in the transcription of target genes (Mukherjee et al., 1987). Genetic studies have consistently demonstrated that common *PPARG* gene variants show profound phenotypic effects such as changes in the lipid profile in adipose tissue and insulin action, leading to human metabolic disease states, obesity, IR, T2DM and cardiovascular diseases (Gurnell, 2003; Heikkinen et al., 2007; Costa et al., 2009).

Table 1.1 Genetic loci associated with insulin resistance

Loci	Disease(s)/Trait(s)	Reference(s)
<i>PPARG</i>	Association with IS, IR and diabetes	Altshuler et al., 2000 Buzetti et al., 2004 Lohmueller et al., 2003 Ludovico et al., 2007 Sanexa et al., 2012
<i>IRS1</i>	Reduced IS Loss of <i>IRS1</i> function Reduced HOMA-IR, high fasting insulin, reduced expression of <i>IRS1</i> protein Decreased IS and increased insulin Adverse metabolic profile	Marini et al., 2003 Almind et al., 1996 Marchetti et al., 2002 Dupuis et al., 2010 Rung et al., 2009 Kilpelainen et al., 2013
<i>GCK</i>	Increased fasting and postprandial blood glucose Increased HOMA-IR Metabolic syndrome traits	Rose et al., 2005 Sparso et al., 2008
<i>GCKR</i>	Decreased fasting insulin, HOMA-IR and T2DM risk, higher TG and lower glucose levels Lower IS and increased C-peptide levels Decreased glucose levels, increased IS, reduced T2DM risk Impaired fasting reduced HOMA-IR, dyslipidaemia and modest reduced T2DM risk	Orho-Melander et al., 2008 Beer et al., 2009 Ingelsson et al., 2010 Orho-Melander et al., 2008 Sparso et al., 2008
<i>IGF1</i>	Associated with fasting insulin and HOM-IR, lower IS and T2DM	Dupuis et al., 2010 Ingelsson et al., 2010

INSR	Reduced expression of both INSR protein and INSR mRNA levels Severe insulin resistance Type A syndrome of extreme insulin resistance	Voight et al., 2010 Raffan et al., 2011 Alzahrani et al., 2012 Moller et al., 1994 Domínguez-García et al., 2014
FTO	Associated with obesity, T2DM, increase in body weight and higher fasting insulin	Frayling et al., 2007 Omori et al., 2008 Voight et al., 2010
KLF14	Primary effect on insulin action, associated with increased diabetes risk	Voight et al., 2010 Wang et al., 2014
GRB14	Attenuate insulin action by inhibiting catalytic activity of INSR	Béréziat et al., 2002
TCERG1L	Associated with increased risk of T2DM Positively associated with both fasting insulin and IR	Chen et al., 2012
SC4MOL	Associated with fasting insulin and IR	Chen et al., 2012

1.10.1.1 *PPARG* domains

There are three splice variants of the *PPARG* (also referred to as PPAR γ) gene namely, PPAR γ 1, γ 2 and γ 3, which differ at their 5-prime ends and are each under the control of their own promoter. The splice variants, PPAR γ 1 and PPAR γ 2 are highly expressed in adipose tissue (Lohman et al., 1998). The *PPARG* protein consists of several functional domains that are used to demonstrate how the different genetic variations impair PPAR γ function (Chandra et al., 2008). The various single amino acid mutations (Table 1.2: p 23) are scattered in the following functional domains of the *PPARG* nuclear receptor: the ligand independent transcriptional activation function domain 1 (AF1); the DNA binding domain (DBD); and a powerful ligand-binding domain (LBD) (Chandra et al., 2008). The DBD facilitates interaction with specific binding sites for peroxisome proliferator response elements (PPREs) in the target gene promoters, while the LBD mediates the heterodimerisation with RXR, which contains the ligand-dependent activation function domain 2 (Chandra et al., 2008).

1.10.1.2 *PPARG* gene: common variants

The Pro12Ala variant occurs in the AF1 domain and can result in reduced transactivation ability and decreased binding affinity of PPAR γ 2 to PPRE on deoxyribonucleotides (DNA) (Deeb et al., 1998; Masugi et al., 2000). Pro115Gln and the rare frame shift mutations, A553 Δ AAAiT and C1984 Δ AG are also in the same AF1 domain. The Pro115Gln variant affects the *PPARG* phosphorylation and activates *PPARG* (Ristow et al., 1998), whilst the frame shift mutations result in truncated proteins in the DNA binding domain. This further affects heterodimer formation and *PPARG* interaction with the target gene promoters (Savage et al., 2003; Knouff & Auwerx, 2004). Cys114Arg, Cys131Tyr and Cys162Trp are variants in the DBD of *PPARG* that encode proteins that are unable to bind to DNA and lack transactivation ability (Agostini et al., 2006).

Table 1.2 *PPARG* common variants associated with insulin resistance and diabetes

Variant(s)	Disease (s)/Trait(s)	Reference(s)
Pro12Ala	Conflicting results of associations with IS, IR and T2DM Meta-analysis associated Pro12Ala variant as risk factor for T2DM Linkage disequilibrium with C1431T No protection from T2DM development Associated with lower insulin levels, lower BMI, higher IS and higher HDL-c levels Reduced weight gain and improved IS Increased BMI, especially in obese subjects Reduced lipolipase activity, leptin, adiponectin and resistin levels Increased leptin Decreased incidence of CVD	Deeb et al., 1998 Altshuler et al., 2000 Willer et al., 2007 Sladek et al., 2007 Tonjes et al., 2006 Zeggini & McCarthy, 2007 Simón et al., 2002 Schneider et al., 2002 Radha et al., 2007 Bidzińska-Speichert et al., 2005 Doney et al., 2004
Pro115Gln	Severe IR in obese individuals	Ristow et al. 1998
Pro495Leu, Val290Met or V318M	Partial lipodystrophy, dyslipidaemia, severe IR, T2DM, fatty liver and hypertension	Barroso et al., 1999 Savage et al., 2003
Phe388Leu	Partial lipodystrophy and dyslipidaemia	Hegele et al., 2002
His447His (silent) or C 1431T or C161T	Increased BMI and fat mass Increased leptin and resistin levels Reduced risk of T2DM	Costa et al., 2009 Meirhaeghe et al., 1998 Valve et al., 1999 Haseeb et al., 2009 Tai et al., 2004 Morris et al., 2012
Arg425Cys, Arg397Cys	Partial lipodystrophy, T2DM	Agarwal & Garg, 2002
A-14G C-681G C689T	Partial familial lipodystrophy, IR and MetS Increased low density lipoprotein (LDL) Increased BMI	Al-Shali et al., 2004 Meirhaeghe et al., 2003 Meirhaeghe et al., 2005
A553AAAAiT C1984AAG	Hyperinsulinaemia, hypertension, T2DM, MetS, dyslipidaemia and hyperlipidaemia	Knouff & Auwerx, 2004 Savage et al., 2003
A-2819G	T2DM and diabetic retinopathy	Costa et al., 2009
Ser289Cys	Colorectal cancer, hypertension, dyslipidaemia and overweight but not T2DM	Capaccio, et al. 2010
Cys114Arg, Cys131Tyr Cys162Trp, Arg357X	Partial lipodystrophy, IR, severe dyslipidaemia low HDL, increased triglycerides	Agostini et al., 2006

The balance of the rare loss-of-function mutations Pro495Leu, Val318Met, Phe388Leu, Arg357X, Arg425Cys and Ser289Cys occur in the LBD of *PPARG*. In the presence of a synthetic ligand, these mutations result in a reduction of *PPARG* transactivation, thereby affecting its ability to recruit cofactors, ligands and RXR alpha (Barroso et al., 1999; Agarwal & Garg, 2002; Hegele et

al., 2002; Savage et al., 2003; Capaccio et al., 2010). In addition, four variants in the *PPARG* putative promoter have also been identified: C-681G, C689T, A2819G and A-14G. Evidence shows associations of these variants with increased low density lipoprotein (LDL) levels and diabetes. A-14G results in decreased activity of the *PPARG* 4 promoter that could affect the expression of some *PPARG* molecular targets (Meirhaeghe et al., 2003; Al-Shali et al., 2004; Meirhaeghe et al., 2005; Costa et al., 2009).

1.10.2 Variants in the *IRS1* gene associated with insulin resistance

1.10.2.1 *IRS1* gene: common variants

Similar to *PPARG*, the *IRS1* gene has various mutations with varying levels of association with obesity, IR and type 2 diabetes mellitus. These have been identified in population studies, as well as in animal models (Sesti et al., 2001; Mercado et al., 2002). The *IRS1* was the first substrate of the insulin receptor substrate (*IRS*) proteins to be identified and localised to human chromosome 2q36-37 (White, 1997). The *IRS1* gene encodes a protein that is phosphorylated by the insulin receptor tyrosine kinase where mutations in this gene are associated with T2DM and susceptibility to insulin resistance (Jellema et al., 2003; Sesti et al., 2001; Sun et al., 1991; Rung et al., 2009).

The most studied mutation in *IRS1* is a relatively infrequent glycine to arginine substitution at position 972 (Gly972Arg variant), which was found to impair insulin signalling in muscle, fat and pancreatic beta cells (Almind et al., 1996; Sesti et al., 2001; Marchetti et al., 2002). The functional role of the *IRS1*, Gly972Arg variant was highlighted when associations with both IR and reduced insulin action were found (Claussen et al., 1995; Sesti et al., 2001; Stumvoll et al., 2001). Carriers of the Arg972 variant showed significantly reduced IS with high values for serum TG, total/HDL-c ratios, FFA levels, systolic blood pressure, microalbuminuria and intima-media thickness. This suggests that the *IRS1* Gly972Arg variant could contribute to the risk for the atherosclerosis/CVD association with diabetes, due to the development of IR-related metabolic abnormalities (Marini et al., 2003). The association of the Gly972Arg variant of *IRS1* with diabetes and its related traits has been investigated in different ethnic groups with conflicting results. In a meta-analysis study conducted by Jellema et al. (2003), the authors reported the Gly972Arg variant to be associated with an increased risk for type 2 diabetes mellitus. However, this association was not found in other large case-control studies (van Dam et al., 2004; Florez et al., 2004; Bezzerra et al., 2004; Zeggini et al., 2004; Morini et al., 2009).

In addition, three variations near the *IRS1* locus have been identified showing evidence of associations with obesity, IR and type 2 diabetes mellitus. In a study by Rung et al. (2009), the SNP rs2943641, located about 547 kb upstream of the *IRS1* gene, was associated with T2DM, IR and

hyperinsulinaemia in a population of French and Danish subjects. The rs2943641 risk allele C showed a reduced HOMA-IR with higher fasting insulin and a lower insulin area under the curve from the oral glucose tolerance test. This allele was also associated with reduced expression of the *IRS1* protein and reduced insulin-induced phosphatidylinositol 3-OH kinase activity in human skeletal muscle (Rung et al., 2009). Similarly, evidence of an association between the SNP rs4675095 in *IRS1* and HOMA-IR was also reported. Although this SNP had too small an effect to pass standard GWAS significance thresholds or in LD with rs2943641, it could possibly be an independent factor associated with the *IRS1* gene (Dupuis et al., 2010). The SNP rs2943650 is located 500 kb upstream of the *IRS1* gene and is an important mediator of insulin and IGF1 signalling. The major allele of rs2943650 was associated with an adverse metabolic profile, and results showed a decrease in body fat percentage, associated with higher TG and lower HDL-c levels. Furthermore, the increased ratio of insulin/glucose area under the curve and the decreased IS were strongly associated with insulin resistance (Kilpeläinen et al., 2013).

1.10.2.2 *IRS* domains

Studies have shown that two regions of the *IRS1* domains are important for interaction with the INSR, namely a pleckstrin homology (PH) domain, located at the NH₂-terminal end of *IRS1* and a phosphotyrosine binding (PTB) domain that is located near the PH domain (Myers et al., 1995; Eck et al., 1996). The *IRS1* PTB domain consists of a compact seven-stranded 3-sheet structure and is capped by a long α -helix at the top end and by a short α -helix at the bottom end. One helical turn that connects with β 4 and β 5 strands, forms the binding site for the NPXY motif of the insulin receptor/s (Eck et al., 1996). The phosphorylation of the NPXY motif of the juxtamembrane domain of the INSR is important because it leads to a recruitment of *IRS1* (Eck et al., 1996; White, 1997). The PH and PTB domains at the N-terminus appear to connect *IRS1* to the activated INSR, with the C-terminal region containing a number of tyrosine phosphorylation motifs that are phosphorylated directly by the insulin receptor/s (Yenush et al., 1996).

1.10.2.3 *IRS1* gene: rare variants

Extensive studies regarding several rare amino acid substitutions of the *IRS1* gene identified the following: Ser892Gly, Ala512Pro, Ala94Thr, Met613Val, Ser1043Tyr, Cys1095Tyr, Ser809Phe, Pro170Arg, Met209Thr, Gly819Arg, Arg1221Cys and the novel T608R (See Table 1.3: p 26).

The functional impact of the Ser892Gly variant *in vitro* has been studied. This variant is located directly upstream to the SH2 binding site of the GRB-2, which links *IRS1* to the mitogenic Ras/MAP kinase pathway (Laakso et al., 1994; Sesti et al., 2001). Overall, a frequency of 0,017% for the Ser892Gly variant was found in diabetic subjects, with similar results also reported in the Finnish, Turks and Mexican American populations. In the Turkish population it was reported that

both the variants Ala512Pro in non-diabetic subjects and Ser892Gly, in diabetic subjects. However, no association with any of these rare variants was found (Funda et al., 2005).

Table 1.3 *IRS1* genes: rare variants associated with human metabolic disease

Variant(s)	Effect on the insulin signalling pathway/disease/trait	Reference(s)
Gly819Arg Arg1221Cys	Involved in the binding of the SHP-2 protein but with no effect on the expression or function of <i>IRS1</i> gene	Imai et al., 1997
Met613Val Ser1043Tyr Cys1095Tyr	No significant associations found with T2DM, BMI, glucose and insulin levels in various populations studied	Sánchez-Corona et al., 2004 Funda et al., 2005
Ser892Gly Ala94Thr Ala512Pro	Located directly upstream to the SH2 binding site of the GRB-2 that links <i>IRS1</i> to the mitogenic RAS/MAP kinase pathway Decreased BMI No associations found with glucose, insulin or BMI for any of these variants	Laakso et al., 1994 Funda et al, 2005 Sánchez-Corona et al., 2004
T608R	May contribute to IR by impairing the metabolic signalling through PI3-kinase-dependent pathways	Esposito et al., 2003
Ser809Phe Pro170Arg Met209Thr	Only found in T2DM individuals Diminishes the binding of <i>IRS1</i> to the insulin receptor due to structural changes in the PTB domain of the <i>IRS1</i> protein Decreased MAP kinase and PI 3-kinase activities	Ura et al., 1996 Eck et al., 1996

The Ala512Pro variant was also found in Danish and French Caucasians but was absent or rare in Finnish, Japanese and South Indian populations (Almind et al., 1993). In Mexican Americans, a modest relationship between the Ala94Thr variant and a decreased BMI, (30,4 kg/m² versus 24,0 kg/m²; p=0,035), was reported (Sánchez-Corona et al., 2004). In Iberian Caucasians, the Ala94Thr and Ser892Arg variants were not detected, but Ala512Pro variant was found in 1/60 non-diabetic subjects (Celi et al., 2000). The mutations Met613Val, Ser1043Tyr and Cys1095Tyr are rare in Mexican Americans (Sanchez-Corona et al., 2004) and absent in the Turkish population (Funda et al., 2005). There were no significant associations reported with any of these variants for diabetes, BMI, glucose and insulin levels. Among Pima Indians, none of these missense mutations were found in either the diabetes or non-diabetes subjects (Celi et al., 1995).

In Japanese T2DM subjects, three additional polymorphisms have been described, namely the Pro170Arg, Met209Thr and Ser809Phe (Ura et al., 1996). The two amino acid substitutions, Pro170Arg (P107R) and Met209Thr (M209T), are located in the PTB domain of *IRS1* and appear

to diminish the binding of *IRS1* to the INSR due to structural changes in the PTB domain of the *IRS1* protein (Eck et al., 1996). The 32D IR cells expressing P170R and M209T showed a reduction in MAP kinase activity due to the decreased binding of *IRS1* to the INSR, together with decreased phosphorylation (Yoshimura et al., 1997). The binding of *IRS1* to the INSR is important for the phosphorylation of *IRS1* (Eck et al., 1996; Yonezawa et al., 1992). Therefore, the reduced phosphorylation of P170R and M209T could be due to the reduced binding to the INSR. In turn, the reduced phosphorylation of *IRS1* could lead to reduced binding sites for SH2 proteins, such as GRB2, SHP-2 and PI 3-kinase, resulting in decreased MAP kinase and PI 3-kinase activities. Although the functional significance of the Ser809Phe *IRS1* variant was not studied *in vitro* it was only found in T2DM subjects (Ura et al., 1996). Whilst in a study with mice, diabetic carriers of Phe809 showed higher HbA1c, fasting glucose and postprandial glucose levels than non-diabetic subjects (Withers et al., 1998).

1.10.3 Glucokinase and glucokinase (hexokinase 4) regulator variants associated with insulin resistance

The common GCKR, P446L variant SNP rs1260326 has been associated with an inverse relationship between glucose and triacylglycerol levels (Orho-Melander et al., 2008). The glucose-lowering leucine allele was also associated with decreased fasting insulin, HOMA-IR, reduction of T2DM risk (Dupuis et al., 2010) and increased triacylglycerol and TC levels (Teslovich et al., 2010). A study of the functional characteristics of the P446L GCKR variant in humans showed the GCKR regulation of GCK was significantly reduced over different concentrations of fructose-6-phosphate (F6P), leading to increased GCK activity that resulted in an increased glycolytic flux and glucose uptake in the liver (Beer et al., 2009). The increased glycolysis could possibly raise the levels of other liver metabolites, and this could explain why variation in GCKR is associated with higher TG and lower glucose levels in humans (Beer et al., 2009).

The SNP rs780094 in GCKR, which is associated with glycaemic traits in the GWAS genetic loci of T2DM, showed a strong association with the minor A allele and hypertriacylglycerolaemia (Diabetes Genetics, 2007; Scott et al., 2007). The minor A allele of rs780094 was also associated with impaired fasting, insulin release, reduced HOMA-IR, dyslipidaemia and a modest decreased risk of type 2 diabetes mellitus (Sparso et al., 2008). This fasting blood glucose-raising allele of GCKR rs780094 was also associated with lower IS and an increased C-peptide level (Ingelsson et al., 2010). The T allele showed a modest association with decreased plasma glucose levels and increased IS that improved IR and reduced the risk of type 2 diabetes mellitus (Scott et al., 2007; Orho-Melander et al., 2008). The index SNP, rs780094, is in strong LD with the missense variant P446L, where the fasting glucose-raising allele inhibits GCK activity in the presence of F6P (Beer et al., 2009), thus leading to increased hepatic glucose production. In another variation in the

pancreatic beta cell promoter of GCK, SNP rs1799884 allele A was significantly associated with increased fasting and post-OGTT levels of circulating glucose, increased HOMA-IR and MetS traits (Rose et al., 2005). Furthermore, results in an additive model reported a significant increase in the fasting serum insulin levels by the G alleles for both the GCK SNP rs1799884 and GCKR SNP rs780094, which could contribute to the difference in metabolic traits observed (Sparso et al., 2008).

1.10.4 *IGF1*: common variant associated with insulin resistance

The SNP rs35767, 1.2 kb upstream of *IGF1* is one of only two loci found to be a genome-wide significant locus similar to the GCKR SNP rs1260326. The G allele of *IGF1*, SNP rs35767, was also associated with fasting insulin, HOMA-IR and type 2 diabetes mellitus (Dupuis et al., 2010; Imamura & Maeda, 2011). In humans with IR and diabetes, *IGF1* SNP rs35767 was also shown to increase IS and lowers the blood glucose (Moses et al., 1996; Clemmons et al., 2005). In another study by Ingelsson et al. (2010), the fasting insulin-raising allele of the *IGF1* SNP rs35767 was associated with lower IS using various indices for the assessment of insulin resistance. Furthermore, mutations in *IGF1* resulted in a deficiency of *IGF1* levels, a condition characterised by reduced growth rate with normal growth hormone levels. Administration of recombinant DNA derived *IGF1* has shown to restore normal growth, with improvement in both glycaemic control and insulin sensitivity (Vaessen et al., 2001; Rietveld et al., 2004; Iñiguez et al., 2006). The *IGF1* SNP rs35767 is possibly another IR locus. This is based on the associations reported with IS, fasting insulin, glycaemic traits and HOMA-IR (Watanabe, 2010; Imamura & Maeda, 2011).

1.11 JUSTIFICATION FOR THE STUDY

The investigations in this dissertation are based on the findings of the larger study that reported a prevalence of diabetes in more than 25% of the mixed-ancestry population of Bellville South, South Africa, with nearly half of the subjects not being aware of their diabetic status (Erasmus et al., 2012). These increasing diabetic figures are not limited to the mixed-ancestry population but are also observed in the black African population, which had previously been thought to be at low risk for cardiometabolic disease traits. For example, since the last study in the 1990s by Levitt et al. (1993) in black South Africans, a marked increase in the prevalence rate of diabetes from 8% to 13,1% was observed (Peer et al., 2012). The increasing diabetes figures around the world are largely attributed to the environmental changes that promote the adoption of unhealthy behaviours and the development of obesity, which in turn are implicated in IR and inflammation and subsequent progression to T2DM and cardiovascular diseases.

The aetio-pathogenesis of T2DM and associated IR is shown to have a strong genetic basis (Brunetti et al., 2014; Povel et al., 2012; Watanabe, 2010). However, in South Africa and Africa in general, genetic abnormalities that can fully account for the increase in obesity and diabetes have not been identified. Therefore, in this study, two key genes that were shown to be associated with diabetes in Caucasians were selected for investigation namely *PPARG* and *IRS1*.

The *PPARG* and *IRS1* loci were identified as candidate genes for IR, as associations between gene variants and impaired IS have been observed which increase the risk of developing type 2 diabetes mellitus. The *PPARG* gene is involved in the regulation of adipocyte gene expression and glucose metabolism, while the *IRS1*Gly972Arg variant alters insulin action. The specific hypothesis behind the proposed investigation is that specific identified sequence variants in the *PPARG* and/or *IRS1* genes, independently or together with obesity, can contribute to the development of IR in a South African mixed-ancestry population with a high incidence of T2DM and CVD risk.

1.12 OBJECTIVES

The specific objectives for the study were as follows:

- the assessment of IR in participants using various surrogate markers of IR e.g. HOMA-IR, fasting insulin resistance index (FIRI), HOMA-β%, glucose and insulin ratio and QUICKI;
- to determine the allele and genotype frequency of the identified sequence variants of *PPARG* and *IRS1* genes;
- to explore the role of specific sequence variants of *PPARG* and *IRS1* genes and their potential associations with either independent or joint effects that may influence IR and may have phenotypic influences on more than one IR Syndrome trait; and
- to investigate IR indices as a clinical tool for improved risk prediction of subclinical CVD/atherosclerosis.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 ETHICS

The sub-study was approved by the Research Ethics Committee of Stellenbosch University (Reference Number: HREC N09/05/146) (Ethics certificate: Appendix A) and the Bellville South Africa Study by the Cape Peninsula University of Technology (CPUT), Health and Wellness Sciences Research Ethics Committee (Reference Numbers: CPUT/HWS-REC 2008/002 and CPUT/HWS-REC 2010). All participants signed consent forms after all the procedures were fully explained to them in the language of their choice. These included consent for their blood samples to be stored and used in future studies, including genetic analysis (Consent form: Appendix B), of which the current study formed a part. Each participant was assigned a unique code that was used for confidentiality purposes, and all data collection documents (Questionnaire: Appendix C) and biological specimen containers reflected this code. Electronic data were password protected, and all documentation was securely locked in fireproof cabinets. The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2 STUDY SETTING AND POPULATION

2.2.1 The Bellville South Africa Study

The Bellville South Africa Study setting, survey design and procedures are described in detail in previous research (Erasmus et al., 2012; Matsha et al., 2013; Zemlin et al., 2011). Briefly, eligible participants were invited to take part in a community-based survey from January 2008 to March 2009 (Cohort 1) and January 2011 to November 2011 (Cohort 2). The sub-study is an extension of the Bellville South Africa Study conducted in Bellville South between 2008 and 2011. Bellville South, a suburb of Cape Town, in South Africa, is a traditionally mixed-ancestry township formed in the late 1950s. According to the 2011 population census, the population of Bellville South was 29 301 with an average household size of 4,84 individuals during the time of the recruitment of participants. Most of the residents in this community have lived there for over five years and in many cases, their entire lives. The socio-economic condition of the people is average, with 37% of households having a monthly income of R3 200 or less. The target population for the Bellville South Africa Study included subjects that were 16 years or older, resulting in a total of 16 168 individuals, of which 14 352 were of mixed-ancestry origin. Of these, 1256 individuals were randomly selected for participation in the Bellville South Africa Study.

2.2.2 The sub-study

The population for the sub-study was selected from the database used in the Bellville South Africa Study and included a total number of 946 participants, comprising of 642 random participants between the ages 35-65 years and 304 voluntary participants, age range 16-95 years, of which 90 were excluded due to missing data, which resulted in 856 participants (235 males and 621 females) consenting to genetic studies. Of the 856 participants, study 1 and 2 combined (Chapter 3) $n = 787$, study 3 (Chapter 4) $n = 820$ and study 4 (Chapter 5) $n = 515$ participants were included in analysis (Figure 2.1: p 32). The diabetes status of the study population was based on a history of doctor-diagnosis, a fasting plasma glucose $\geq 7,0$ Mmol/L and/or a 2-hour post-oral glucose tolerance test with plasma glucose $\geq 11,1$ Mmol/L. This study was conducted at the Department of Biomedical Sciences, CPUT and the Division of Chemical Pathology, Tygerberg Hospital at Stellenbosch University, South Africa.

2.2.2.1 Selection criteria for the study population

Inclusion criteria

- South African citizens of mixed-ancestry origin
- age group 16 to 95 years
- participants who consented to genetic analysis

Exclusion criteria

- acute illness
- other race than mixed ancestry participants
- participants that did not consent to genetic analysis
- participants with incomplete information on the database
- pregnant females

2.3 DATA COLLECTION AND MANAGEMENT

Data was captured on an Excel spreadsheet, and the statistical package, R (version 3.0.0 {2013-04-03}, the R Foundation for Statistical Computing, Vienna, Austria) was used. See Section 2.7 for the statistical analysis used in this study.

2.3.1 Questionnaire

To obtain the medical information and samples of the participants, a detailed protocol describing the data collection procedures (questionnaires and physical examinations) was developed. The team members consisting of professional nurses and field workers were trained, and a pilot study in a neighbouring community with similar demographics was performed to validate the questionnaire

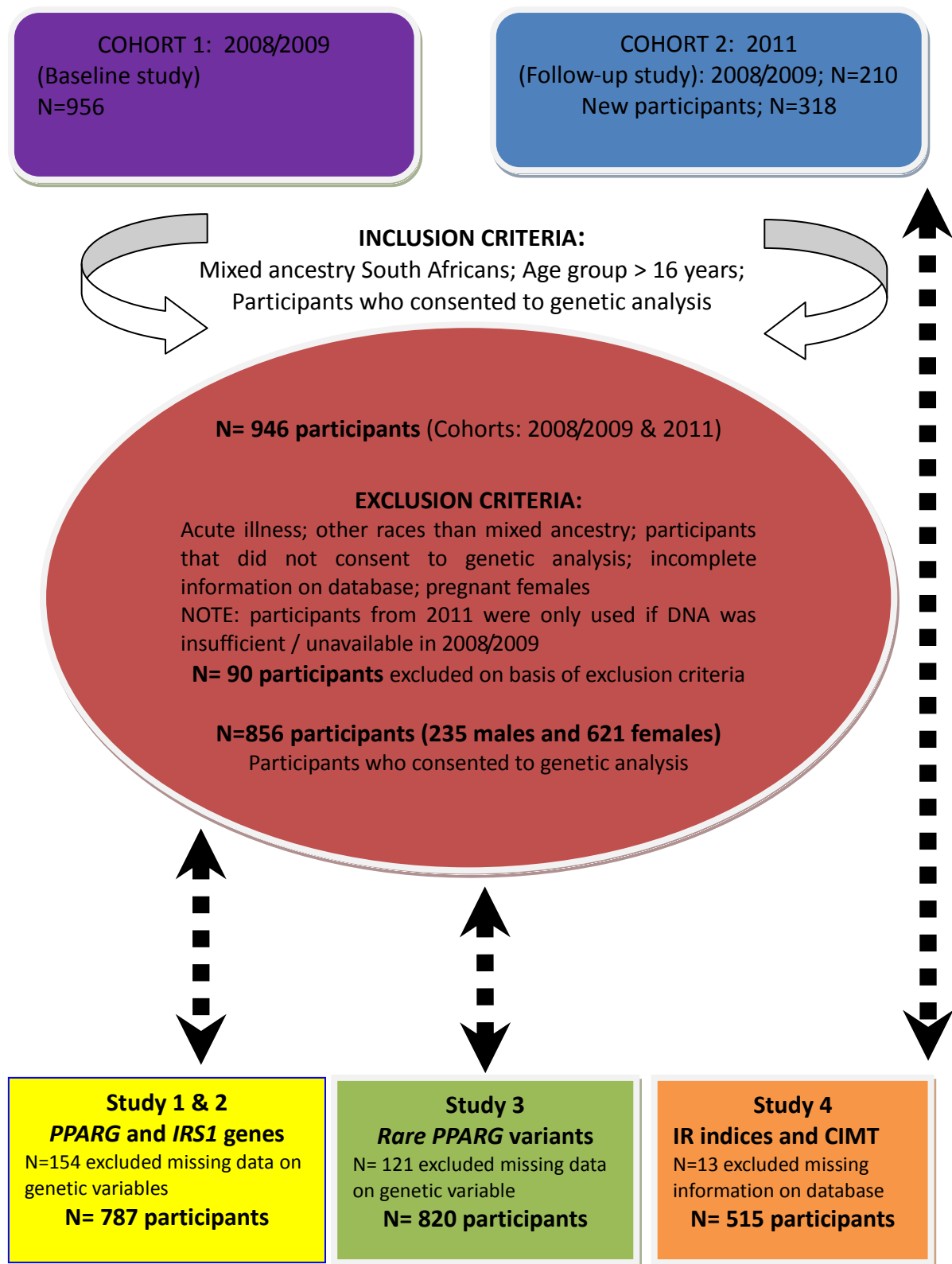


Figure 2.1 Flow-diagram of the population for the sub-study selected from the Bellville South Africa Study database.

Abbreviations: Carotid Intima Media Thickness (CIMT), Insulin Receptor Substrate (*IRS*) 1, Peroxisome proliferator-activated receptor gamma (*PPARG*), Insulin Resistance (IR)

and synergise the workflow. A supervisor was allocated to each team to monitor the performance of the personnel and calibrate equipment according to a standard protocol. In addition, weekly meetings were held to assess progress, solve problems and re-train the research team (if necessary). A questionnaire, designed to retrospectively obtain information on lifestyle factors, such as smoking and alcohol consumption, physical activity, diet, family history of CVD and T2DM and demographics, was administered by trained personnel (Questionnaire: Appendix C). The questionnaire was adapted from several existing standards and recognised sources (Bradshaw et al., 1995; Ewing, 1984) and was pre-tested in a neighbouring community with similar demographics. Information regarding medication taken by participants was obtained through clinic cards and records that participants brought to the study site. The more detailed the information retrieved, the more accurate and complete the database, allowing for more association tests during the statistical analysis.

2.3.2 Anthropometric and metabolic parameters

Professional nurses and field workers trained in the use of prescribed standardised techniques conducted anthropometric measurements and biochemical analyses. These standardised techniques and associated data-collection methods were piloted and used in a research project aimed at investigating the prevalence of obesity, diabetes, metabolic syndrome and cardiovascular risk among adults in the Bellville South, Western Cape Province. In the pilot study, a careful selection of instruments with adequate detection limits and sensitivity was carried out to enhance the accuracy and validity of results. Statistical measures in the form of repeated measures were used to ensure inter- and intra-validity. Measurements and analyses were performed, as detailed in the sections below. The principles and performance characteristics of assays used in this dissertation are summarised in Appendix D.

2.3.2.1 Anthropometric measurements

Weight measurements were obtained using a digital bathroom electronic scale (Fuzhou Sunny Electronic Co., Ltd., China). All heavy clothing and shoes were removed. The scale was calibrated and standardised using a weight of known mass. The subjects were asked to stand in the middle of the scale platform after the scale had been zeroed. It was ensured that the subjects' weight was evenly distributed with the arms hanging relaxed along their sides. Readings were taken to the nearest 0,1 kg. Height was measured using a stadiometer (Anand Medical Exports, India). The subject was asked to stand on a flat surface that was at a 90° angle to the vertical lever/board of the stadiometer. The scapula and the buttocks were in contact with the wall/ board, with the buttocks and the heels in the same vertical line. The subject was then asked to take a deep breath while maintaining a fully upright position. The required accuracy was 0,1 cm (Lohman, Roche, Martorell, 1988). The BMI was calculated for each subject as: $\text{weight (kg)} \div [\text{height (m)}]^2$.

The waist measurement was taken with the subject in an erect position, abdomen relaxed, arms at the sides and feet together. This measurement was performed facing the subject in a horizontal plane, with a non-elastic tape measure placed at the level of the natural waist. The natural waist is defined as the narrowest part of the torso as seen from the anterior view. In obese subjects, it may be difficult to see the waist narrowing and therefore, the smallest circumference measured in the area between the ribs and the iliac crest was taken. The measurement was taken three times at the end of a normal expiration and the average recorded. The required accuracy was 0,1 cm. The hip circumference was measured as the maximal circumference over the buttocks, using a non-elastic tape. The field worker had to squat beside the subject so that the maximum extension of the buttock in the horizontal plane at this level could be taken without compressing the skin. The measurement was taken three times, and the average of the measurements recorded. The required accuracy was 0,1 cm. The waist-to-hip ratio was calculated as the average of the waist circumference divided by the average of the hip circumference. This was recorded to four decimal places.

2.3.2.2 Blood pressure

Blood pressure measurements were carried out according to the World Health Organization (WHO) guidelines (Chalmers et al., 1999). Measurements were performed using a semi-automatic digital blood pressure monitor (Rossmax PA, USA) on the right arm, with the participant in a sitting and relaxed position, and not having ingested coffee or smoked for 30 minutes before the measurement. The cuff was placed at a point midway between the olecranon and acromion to ensure an accurate measurement. After a ten-minute rest period, three readings were taken at five-minute intervals, and the lowest of the three readings was taken as the blood pressure.

2.3.2.3 Biochemical analysis

Fasting blood samples were used for all the biochemical measurements. Prior to screening, the fasting state was determined in an interview on the morning of the examination. Participants with no history of doctor-diagnosed diabetes mellitus underwent a 75 g OGTT, as recommended by WHO (Alberti & Zimmet, 1998). Plasma glucose was measured by an enzymatic hexokinase method, glycated haemoglobin (HbA1c) by a turbidimetric inhibition immunoassay, and the creatinine measurement was obtained by using the standardised creatinine assay (Cobas 6000, Roche Diagnostics, Germany). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG) and γ -glutamyltransferase (GGT) were estimated by enzymatic colourimetric methods (Cobas 6000, Roche Diagnostics). The glomerular filtration rate (GFR) was estimated by the 4-variable Modification of Diet in Renal Disease (MDRD) equation (Levey et al., 1999; Levey et al., 2006) applicable to standardised serum creatinine values. Low-density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (Friedewald et al., 1972.). Insulin

was determined by a micro-particle enzyme immunoassay (AxSYM, Abbott). C-reactive protein was measured by a high-sensitivity CRP assay, based on the highly sensitive Near Infrared Particle Immunoassay rate methodology (Image® Immunochemistry System; Beckman Coulter), with a lower limit of detection of 0,2 mg/L.

2.4 ASSESSMENTS OF INSULIN RESISTANCE/SENSITIVITY

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: $\text{HOMA-IR} = [\text{fasting insulin concentration (mIU/L)} \times \text{fasting plasma glucose (mM)}] / 22,5$; while homeostasis model assessment of functional beta-cells (HOMA- $\beta\%$) were estimated using the formula: $20 \times \text{fasting insulin (mIU/L)} / \text{fasting glucose (mM)} - 3,5$. The fasting insulin resistance index (FIRI) was calculated with the formula: $[\text{fasting insulin (mIU/L)} \times \text{fasting glucose (mM)}] / 25$ and the quantitative insulin sensitivity check index (QUICKI) as: $1 / [\log (\text{fasting insulin (mIU/L)}) \times \log (\text{fasting glucose (mM)})]$.

2.5 CAROTID INTIMA MEDIA THICKNESS

2.5.1 Carotid artery ultrasound protocol

Two qualified sonographers measured carotid intima media thickness (CMT) in longitudinal section at the far wall of the distal common carotid arteries, 2 cm from the bifurcation, at three consecutive end-points, 5 mm to 10 mm apart. Carotid intima media thickness was reported in millimetres and used as a measure of subclinical CVD/atherosclerosis. The ultrasound examination was performed according to the 'As Low As Reasonably Achievable' (ALARA) principle to avoid biological effects that may be associated with ultrasound. The exposure output of the machine was kept to a low value with the thermal index <1 , which still resulted in an accurate diagnosis (Kremkau, 2006). The mean of six readings (three from each side) was calculated for each participant using a portable B-mode and spectral Doppler ultrasound scanner equipped with cardiovascular imaging software. The GE LOGIQ e® (General Electric Healthcare, Germany) high-performance multipurpose colour compact ultrasound system included new imaging CrossXBeam technologies, with multi-frequency virtual apex on the phased array cardiac transducer (3S-RS wide band phased probe 1.7-4.0 MHz) for echocardiography, and a linear wide-band vascular transducer (8L-RS 4.0-12 MHz linear probe) to improve diagnostic confidence and imaging clarity for the carotids.

2.6 GENETIC ANALYSES

Samples were genetically analysed, initially with the extraction of DNA from whole blood, followed by genotyping and subsequently, automated sequencing.

2.6.1. DNA extraction

Venous blood samples were collected from consenting adults by professional medical nurses in specimen tubes containing ethylenediaminetetraacetic acid (EDTA). The samples were transported to the laboratory at room temperature and stored at -20°C if immediate extraction was not possible. Genomic DNA was extracted from the venous blood samples using a modified salting out procedure (Miller et al., 1988). A volume ranging from 5-10 mL of whole blood was transferred from an EDTA tube into a 50 mL Falcon tube. Cold lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0,1 mM EDTA, pH 7,4) was added, according to the volume of blood used (30 mL of cold lysis buffer for every 10 mL of blood), to produce a final volume of 40 mL. All reagents used in the subsequent steps were adjusted accordingly. The mixture was then placed on ice for 15 minutes and inverted every five minutes. This mixture was centrifuged at 1500 rpm (400 x g) for 10 minutes at 4°C (J-6M/E centrifuge, Beckman, United Kingdom). The supernatant was carefully discarded and the pellet (white blood cells) resuspended in 0,9% phosphate buffered saline (PBS) (2,68 mM KCl, 136,89 mM NaCl, 6,46 mM Na₂HPO₄·2H₂O, 1,47 mM KH₂PO₄, pH 7,4). This was followed by centrifugation at 1500 rpm for 10 minutes, after which the supernatant was discarded and the pellet resuspended in nuclear lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, pH 8,2), 0,3 mg/mL Proteinase K and 1% (w/v) sodium dodecyl sulphate (SDS). The contents were mixed well and incubated at 55°C overnight. Thereafter, 6 M NaCl was added to the solution, and the tubes were shaken vigorously for one minute. The mixture was centrifuged at 2500 rpm (1500 x g) for 30 minutes. The supernatant containing the DNA was transferred to a clean Falcon tube and the pellet discarded. The supernatant was vortexed for 15 seconds, followed by centrifugation at 2500 rpm (1500 x g) for 15 minutes. This supernatant was transferred to a clean Falcon tube without the foam or the pellet. Two volumes of cold 99,9% (v/v) ethanol (C₂H₆O) was added to each tube and agitated to precipitate the DNA. The DNA was removed using a sterile pipette tip, placed in a clean 1,5 mL Eppendorf tube and washed with cold 70% (v/v) ethanol. The tube was centrifuged using a benchtop microcentrifuge (Microcentrifuge® Lite, Beckman Coulter™) at 8000 rpm (6000 x g) for two minutes. The 'washing step' was repeated until the pellet was clear. The ethanol was discarded and the tube left at room temperature to dry. Depending on the size of the pellet, 200-800 µL of Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA) was added to dissolve the DNA. To ensure a homogenous solution the DNA was dissolved by shaking the tubes using a rotator at room temperature.

2.6.1.1 Purity of DNA

The isolated DNA was quantified in terms of the DNA concentration and purity by using the Nanodrop® ND-100 Spectrophotometer v3.0.1 (NanoDrop Technologies Inc, DE 19810 USA). The NanoDrop® UV/VIS spectrophotometer was employed to determine accurately the nucleic acid concentration in a sample, which was recorded in nanograms per microlitre (ng/µL). Where

possible, the DNA samples were diluted to obtain a final concentration of 200 ng/μL. The quality and purity of the DNA were determined by measuring the ratio of absorbance at 260 nm and 280 nm, whereby a ratio of approximately 1,8 to 2,0 was considered to be of good quality.

2.6.2 Molecular analysis of the Single Nucleotide Polymorphisms

The SNPs were genotyped using the real-time polymerase chain reaction (RT-PCR). The conventional polymerase chain reaction (PCR) was used to validate genotypes that could not be determined by RT-PCR, by the sequencing of the amplified PCR products. Positive controls (samples with known genotypes) were identified for both methods.

2.6.2.1 Real-time polymerase chain reaction

Real-time polymerase chain reaction assays were used for SNP genotyping according to the manufacturer's instructions (TaqMan genotyping assay; Applied Biosystems, South Africa). The assays were carried out using the BioRad MiniOpticon Real time PCR system (BioRad) and corresponding software (CFX Manager). TaqMan genotyping combines hybridisation and 5' nuclease activity of polymerase coupled with fluorescence detection. This method utilises four oligonucleotides: two allele-specific probes and a pair of PCR primers flanking the region containing the polymorphism of interest. A reaction mix was produced to contain 5 μL TaqMan Universal PCR Master Mix, 0,25 μL Assay Mix, 1-3 ng DNA template and distilled water to a final volume of 10 μL. For each run, a positive control (any of the samples with a known genotype according to the sequencing results) was included, as well as a minimum of three non-template controls.

The allele-specific probes carry a fluorescent reporter dye at one end and a non-fluorescent quencher at the other end. The probes contained different fluorescent reporter dyes, including VIC and FAM to differentiate between the amplification of each of the alleles. VIC has an emission maximum of 554 nm, thus emitting a green-yellow colour and in this study, was used to measure the minor allele presence or absence. FAM, 6-carboxyfluorescein, is the most commonly used fluorescent dye and fluoresces blue in colour at 517 nm. During the extension stage of PCR, the polymerase enzyme only cleaves on the hybridised probe that is perfectly matched, thus freeing the reporter dye from the quencher. This generates the fluorescence signal in the absence of the quencher. On the other hand, the mismatched probe remains intact and shows no fluorescence. This allows detection of both alleles during the reaction based on the hybridisation of the respective probes. The signal of the dye is dependent on the amount bound to the double-stranded DNA, thus increasing proportionally with the generated quantity of amplicons, with a linear increase until a plateau is reached. An amplification plot was used to display the fluorescence over the cycles.

For allelic discrimination, the fluorescence results were viewed once the cycles were completed, hence, endpoint quantitative-PCR.

2.6.2.2 Polymerase chain reaction

The success of PCR assays are dependent on the quality of the primer sets. Standard guidelines were followed for designing primers, with special attention to appropriate melting temperatures, primer lengths, GC content, hairpin loops and the complementarity of the primers. The primers were designed from the reference nucleotide sequence flanking the polymorphisms of interest (Primers designed : Appendix E). Free available primer design programmes such as Primer3plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>) and Integrated DNA Technologies (<http://eu.idtnda.com>) were used for designing the primer sets. The primers were synthesised by Integrated DNA Technologies and supplied by White Head Scientific (Cape Town, South Africa). The primers were rehydrated according to the manufacturer's instructions: a stock solution of 100 μ M was prepared in nuclease-free water, allowed to stand at room temperature for four hours to dissolve, after which it was further diluted to a 20 μ M working solution. A master mix was made up to contain 5-10 μ g DNA template, 0,24 μ M of both forward and reverse primers, 1 x buffer, 1,0 mM magnesium chloride (MgCl_2), 0,3 μ M dNTP, 1,25 μ L DNA polymerase, and distilled water was added to yield a final volume of 50 μ L reaction mixture. Both the peroxisome proliferator-activated receptor gamma (*PPARG*) and insulin receptor substrate (*IRS*)1 gene variants were amplified in a Perkin Elmer 2720 thermal cycler (Applied Biosystems, USA). This included initial denaturation at 95°C for three minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C–55°C for 45 seconds depending on the primer set used (detailed in Appendix E.), extension at 72°C for 45 seconds and final extension at 72°C for five minutes.

Amplicons, which ranged from 200 to 380 base pairs, were electrophoresed on 2% agarose gel and visualised in a GelDoc system (BioRad, GmbH, South Africa). The gel was prepared by weighing 2 g of agarose powder (White Head Scientific, South Africa) and dissolving it in 100 mL of 5 mM di-sodium boratedecahydrate buffer, pH adjusted to 8,5. The low conductivity of di-sodium boratedecahydrate buffer allows gels to be run at a high voltage and speed, thereby separating the DNA fragments faster. The solution was heated in a microwave oven until the powder was completely dissolved. The solution was allowed to cool without solidifying before adding 0,1% ethidium bromide. This was poured into a casting tray (7 x 15 cm) with combs inserted to create wells and allowed to set at room temperature before loading the samples. Amplicons (8 μ L each) were mixed with 5 μ L of 6 x loading buffer (Fermentas, Cape Town, South Africa) and loaded onto the gel. A 100-base pair DNA molecular marker was run simultaneously as a marker. The gels were run at 140 volts for 30 minutes and viewed under the GelDoc XR System (Bio-Rad Laboratories, Cape Town, South Africa).

2.6.2.3 Sequencing

For sequencing, the PCR product was purified using Exonuclease I and Shrimp Alkaline Phosphatase (Inqaba Biotechnologies, South Africa) according to the manufacturer's instructions. This was performed by adding 5U of Shrimp Alkaline Phosphatase and 5U of Exonuclease I to 5 μ L of the PCR product. The mixture was incubated at 37°C for 15 minutes, followed by heating at 80°C for 15 minutes to deactivate the enzymes. The concentration and purity of the PCR product was checked using the Nanodrop® according to the manufacturer's specifications. Where necessary, samples were diluted to the desired concentration using nuclease-free water. Purified amplicons were sequenced using a BigDye terminator version 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions and resolved on an ABI 3130X ® Genetic Analyzer (Applied Biosystems, USA) at the Central Analytical Facility, Stellenbosch University. The sequencing data were further analysed using the Geospiza's FinchTV version 1.4 software programmes.

2.7 STATISTICAL ANALYSIS

Data are presented as mean (standard deviation, SD) or median (25th-75th percentiles) for continuous variables and as count and percentage for categorical variables. For group (sex, diabetes status) comparisons, the chi square test and student t-test were used. Where necessary, traits were log-transformed to approximate normality prior to analysis. The SNPs were tested for departure from the Hardy-Weinberg Equilibrium (HWE) expectation via a chi square goodness of fit test. All analyses used the statistical package R (version 3.0.0 {2013-04-03}, the R Foundation for Statistical Computing, Vienna, Austria). The R package genetics was used for genetic frequency distributions, allelic association, HWE and linkage disequilibrium (LD) testing. The HWE describes the equilibrium at a single locus in a randomly mating population (Gillespie, 2004). HWE frequencies detail unchanged genotype proportions in a large population from generation to generation, providing there are no evolutionary forces such as mutation, migration and selection (Reviewed by Lewis & Knight, 2012). It allows for the prediction of genotype frequencies from the knowledge of gene frequencies. LD measures the correlation between SNP alleles at sites in the same region of the genome. Many different statistical measures to quantify LD between two SNPs have been proposed, with D' and r^2 being the most widely used (Devlin & Risch, 1995). Linear regression models were used for the analysis of quantitative traits and logistic regression models for dichotomous traits, always assuming additive models for the SNPs. Using linear and logistic models enabled the adjustment of all analyses for the known confounders, as specified in the results.

The additive allelic association of each SNP with each trait, overall and according to the T2DM status, was investigated and tested for heterogeneity by adding the interaction term of T2DM and each SNP to a model that contained the main effects of T2DM and the relevant SNP. The interaction term is defined as the interdependence of effects at two loci. If the disease risk caused by the presence of risk alleles at two loci can be inferred from the marginal effects of the presence of each risk allele individually, then no interaction is present. When the joint effects of risk alleles at both loci are much larger (or smaller) than implied by the marker-specific effects, then interaction exists (Reviewed by Lewis & Knight, 2012). The continuous associations between CIMT and the indices were assessed graphically with the use of a correlation matrix before and after applying the Box-Cox power (Box & Cox, 1964) transformations to improve the shape of the associations. The 'Covariance Estimation for Multivariate t Distribution' method was then used to derive the correlation coefficients, while minimising the potential effects of outliers (Kent et al., 1994). The Steiger t-test was used to compare correlation coefficients among indices. Regression coefficients used to indicate the size of the association of each of the indices with CIMT was derived from robust multiple linear regression models that included each of the four variables of interest: age, sex, BMI and diabetes status. The significance level was set at 0,05.

CHAPTER 3

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA Pro12Ala INTERACTS WITH THE INSULIN RECEPTOR SUBSTRATE 1 Gly972Arg AND INCREASES THE RISK OF INSULIN RESISTANCE AND DIABETES IN A MIXED-ANCESTRY POPULATION FROM SOUTH AFRICA

3.1 BACKGROUND

Insulin resistance is a fundamental aetiopathogenic factor for T2DM and is also linked to a wide array of other pathophysiological derangements including hypertension, hyperlipidaemia, atherosclerosis and polycystic ovarian disease (Reaven, 1997). The gold standard method for assessing IR/S is the euglycemic hyperinsulinemic clamp (Polonsky et al., 1988; DeFronzo et al., 1979). However, this technique is cumbersome, particularly for large-scale epidemiological studies. Thus, relatively simple, non-invasive alternative techniques validated against the euglycemic clamp have been proposed. The homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al., 1985) and quantitative insulin sensitivity check index (QUICKI) (Katz et al., 2000) methods are commonly used for IR and IS respectively. It is well recognised that the development of IR and T2DM is, in part, modulated by the gene-gene interaction processes.

The peroxisome proliferator-activated receptor gamma (*PPARG*) and the insulin receptor substrate (*IRS*)1 genes have been shown to be associated with both IR and type 2 diabetes mellitus (Buzetti et al., 2004; Ludovico et al., 2007; Gouda et al., 2010; Trombetta et al., 2013; Jellema et al., 2003; Sesti et al., 2001). The *PPARG* is a member of the super family of nuclear receptors reported to be involved in the regulation of adipocyte differentiation (Evans et al., 2004), lipid metabolism and insulin sensitivity (Buzetti et al., 2004). Several variants in the *PPARG* gene have been identified, with the most prevalent variant being the Pro12Ala polymorphism, resulting from the CCA-to-GCA missense mutation in codon 12 of exon B that encodes the NH₂ terminal residue (Auwerx, 1999; Vigouroux et al., 1998; Stumvoll & Haring, 2002). This polymorphism has been shown to produce a protective effect against IR and type 2 diabetes mellitus (Trombetta et al., 2013; Stumvoll & Haring, 2002; Mori et al., 2001). On the other hand, the most common polymorphism in the *IRS1* gene is the Gly972Arg variant located near the *src* homology 2 (SH2) protein binding sites between the two consensus motifs Y⁹³⁹MKM and Y⁹⁸⁷MTM. These potentially bind to the p85 subunit of PI 3-kinase (Yoshimura et al., 1997).

Insulin produces most of its metabolic actions via the PI 3-kinase pathway, an enzyme that promotes translocation of glucose transporter proteins, glycogen and lipids. It is also involved in protein synthesis, anti-lipolysis and the control of hepatic gluconeogenesis. The substitution of Gly (GGG) for Arg (AGG) can result in a conformational change in these binding sites, which may impair the association of *IRS1* with the PI 3-kinase p85 regulatory unit (Schmitz-Peiffer & Whitehead, 2003; Pederson, 1999; Porzio et al., 1999). Cells expressing G971R have shown a reduction in PI 3-kinase activity due to a reduced association with PI 3-kinase (Yoshimura et al., 1997). Studies *in vitro* have shown that the Arg972 allele leads to a loss of the *IRS1* function, thereby impairing insulin signalling in several target tissues, e.g. muscles, fat and pancreatic beta cells. However, studies *in vivo* have reported associations between the *IRS1* Gly972Arg variant with both IR and reduced insulin secretion (Sesti et al., 2001; Almind et al., 1993; Marchetti et al., 2002). In contrast, the glycine to arginine substitution in codon 972 (Gly972Arg) of the *IRS1* gene was associated with an increased risk of insulin resistance (Burguete-Garcia et al., 2010). In view of the above, an investigation was carried out into the independent and joint effects of *PPARG* Pro12Ala and *IRS1* Gly972Arg on markers of IR and T2DM in the mixed-ancestry population of South Africa, a population with an elevated risk of type 2 diabetes mellitus.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 Ethical approval, consent and confidentiality

The study setting, survey design and procedures have been described in detail in previous research (Erasmus et al., 2012; Matsha et al., 2013; Zemlin et al., 2011) as well as in chapter 2. Briefly, eligible participants were invited to take part in a community-based survey from January 2008 to March 2009 (Cohort 1) and January 2011 to November 2011 (Cohort 2). The study was approved by the Research Ethics Committee of Stellenbosch University (HREC Reference Number: N09/05/146) and the CPUT, Faculty of Health and Wellness Sciences Ethics Committee (Reference Numbers: CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed consent forms after all the procedures were fully explained in the language of their choice.

3.2.2 Clinical data

All consenting participants received a standardised interview and physical examination during which their blood pressure was measured according to the World Health Organization (WHO) guidelines (Chalmers et al., 1999) using a semi-automated digital blood pressure monitor (Rossmax PA, USA) on the right arm, with the participant in a sitting position. Other clinical measurements included the body weight, height, waist and hip circumferences. Weight (to the

nearest 0,1 kg) was determined with the subject wearing light clothing and without shoes and socks, using a Sunbeam EB710 digital bathroom scale, which was calibrated and standardised using a weight of known mass. The waist circumference was measured using a non-elastic tape at the level of the narrowest part of the torso, as seen from the anterior view. The hip circumference was also measured using a non-elastic tape around the widest portion of the buttocks. All anthropometric measurements were performed three times and their average used for analysis. Participants with no history of doctor-diagnosed diabetes mellitus underwent a 75 g OGTT, as recommended by the WHO (Alberti & Zimmet, 1998).

3.2.3 Laboratory measurements

Blood samples were collected after an overnight fast and processed for further biochemical analysis. Plasma glucose was measured by the enzymatic hexokinase method (Cobas 6000, Roche Diagnostics, Germany) and glycated haemoglobin (HbA1c) by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics, Germany), this being a National Glycohaemoglobin Standardisation Programme (NGSP) certified method. Creatinine levels were measured using the standardised creatinine assay (Cobas 6000, Roche Diagnostics, Germany). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG) and γ -glutamyltransferase (GGT) were estimated by enzymatic colourimetric methods (Cobas 6000, Roche Diagnostics). Low-density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (Friedewald et al., 1972). Insulin was determined by a microparticle enzyme immunoassay (AxSYM, Abbot). C-reactive protein was measured by a high-sensitivity CRP assay, based on the highly sensitive Near Infrared Particle Immunoassay rate methodology (Image® Immunochemistry System; Beckman Coulter), with a lower limit of detection of 0,2 mg/L.

3.2.4 Single Nucleotide Polymorphisms genotyping

Genomic DNA was extracted from whole blood samples collected in an EDTA tube. Single nucleotide polymorphisms in the *IRS1* (rs1801287, G>A) and *PPARG* (rs1801282, C>G) were genotyped using a high-throughput real-time polymerase chain reaction. This was carried out in two independent laboratories on the ABI Prism 7900HT platform (Applied Biosystems, USA) and a BioRad Optica (BioRad, USA), using the Taqman genotyping assay (Applied Biosystems, USA). Direct sequencing was used for the analytical validation of the high-throughput genotyping as the gold standard.

3.2.5 Definitions and calculations

Body mass index was calculated as weight per square metre (kg/m^2) and waist-hip-ratio (WHR) as waist/hip circumferences (cm). The T2DM status was based on a history of doctor diagnosis, a fasting plasma glucose $\geq 7,0$ Mmol/L and/or a 2-hour post-OGTT plasma glucose $\geq 11,1$ Mmol/L.

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: $\text{HOMA-IR} = [\text{fasting insulin concentration (mIU/L)} \times \text{fasting plasma glucose (Mmol/L)}] / 22,5$; while homeostasis model assessment of functional beta-cells ($\text{HOMA-}\beta\%$) were estimated using the formula: $20 \times \text{fasting insulin (}\mu\text{IU/mL)} / \text{fasting glucose (Mmol/mL)} - 3,5$. The fasting insulin resistance index (FIRI) was calculated with the formula: $[\text{fasting insulin (}\mu\text{U/mL)} \times \text{fasting glucose (mM)}] / 25$ and the quantitative insulin-sensitivity check index (QUICKI) as: $1 / [\log (\text{fasting insulin (}\mu\text{U/mL)}) \times \log (\text{fasting glucose (mg/dL)})]$. Glomerular filtration rate (eGFR) was estimated by the 4-variable modification of diet in renal disease (MDRD) equation (Levey et al., 1999; Levey et al., 2006) applicable to standardised serum creatinine values.

3.3 STATISTICAL ANALYSIS

The general characteristics of the study group are summarised as count and percentage for dichotomous traits, mean and standard deviation, (SD) or median and 25th-75th percentiles for quantitative traits. Where necessary, traits were log-transformed to approximate normality prior to analysis. The SNPs were tested for departure from the Hardy Weinberg (HWE) expectation via a chi square goodness of fit test. Linear regression models were used for the analysis of quantitative traits and logistic regression models for dichotomous traits, always assuming additive models for the single nucleotide polymorphisms. Using linear and logistic models enabled the adjustment of all analyses for known confounders, as specified in the results. The additive allelic association of each single nucleotide polymorphism with each trait, overall and according to T2DM status, was investigated and tested for heterogeneity by adding the interaction term of T2DM and each single nucleotide polymorphism to a model that contained the main effects of T2DM and the relevant single nucleotide polymorphism. Results corresponding to p-values below 5% are described as significant. There was no adjustment for multiple testing. All analyses used the statistical package R (version 3.0.0 {2013-04-03}, the R Foundation for statistical computing, Vienna, Austria).

3.4 RESULTS

Of the 946 participants in the survey, 941 consented for genetic studies. Among the latter, 154 were excluded due to missing data on the genetic variables. Therefore, 787 had valid data for the analyses. The clinical characteristics of the participants overall and according to their T2DM status are summarised in Table 3.1 (see p 48), indicating that 212 participants (26,9%) had type 2 diabetes mellitus. As expected, the distribution of the levels of IR/S indicators was significantly different between the two groups (all $p < 0,0001$, except for the glucose/insulin ratio ($p = 0,016$)). Furthermore, compared with non-diabetic participants, those with T2DM had significantly higher levels of adipometric variables (all $p \leq 0,028$), systolic blood pressure ($p < 0,0001$), TG ($p < 0,0001$), GGT and CRP (both $p < 0,0001$), whilst eGFR ($p = 0,015$) and HDL-c ($p = 0,0001$) were significantly lower.

The *IRS1*, Gly972Arg and *PPARG*, Pro12Ala variants were in HWE ($p > 0,05$), and their genotype and allele distribution by T2DM status is summarised in Table 3.2 (see p 49). Overall, the genotype distributions of the two polymorphisms did not differ significantly between the two groups. However, the allele G of *PPARG* (12Ala) was significantly more frequent in the diabetic subjects than in the non-diabetic subjects (13,7% vs. 9,3%, $p = 0,012$). The genotype frequencies, *PPARG* Pro12Ala and *IRS1* Gly972Arg were 10,4% and 7,7% respectively.

In the generalised linear regression analyses, which were adjusted for age, sex and T2DM (Table 3.3: p 50), the *IRS1* allele A (972 Arg) was not associated with the markers of glycaemia, IR or IS, both overall and in participants with and without T2DM who were analysed separately. There was no evidence of a significant statistical interaction by T2DM status (all interaction $p \geq 0,330$), except in the 2-hour glucose analysis, in which the size of the effect appeared to be greater, although non-significantly, among the diabetic participants (interaction $p = 0,038$). In similar generalised linear regression models (Table 3.3: p 50), the *PPARG* allele C (Pro12) increased the 2-hour insulin levels in the overall cohort ($p = 0,009$) and only increased in the non-diabetic group ($p = 0,0003$) after stratification by T2DM status, with a significant statistical interaction ($p = 0,017$). Otherwise, the *PPARG* C allele was not significantly associated with the marker of glycaemia, IS or IS, both overall and by T2DM status. However, there is evidence that the effect on the 2-hour glucose could be more pronounced in people with T2DM ($p = 0,002$) for the *PPARG* C allele T2DM interaction. The main effects for *IRS1* and *PPARG* did not change significantly when they were adjusted for each other in the regression models, with or without further adjustment for their interaction term.

In the logistic regression models adjusted for each other, or containing age and sex, and with and without further adjustment for markers of IR/S (Table 3.4: p 51), neither the *IRS1* allele A nor the *PPARG* was significantly associated with prevalent type 2 diabetes mellitus. However, in the model containing both alleles and their interaction term, the *PPARG* allele C was significantly associated with a higher risk of prevalent T2DM, odds ratio (95% confidence interval) 1,64 (1,00-2,64).

3.5 DISCUSSION

The mixed-ancestry population of South Africa has one of the highest prevalence of T2DM in South Africa and Sub-Saharan Africa at large. However, genetic abnormalities that can fully account for this have not been identified. In this study, it is shown that *PPARG* Pro12 was significantly associated with IR and T2DM in this population. It was observed that neither the *IRS1* 972Arg allele nor *PPARG* 12Ala were associated with T2D or IR/S, but in a model containing both the alleles and their interaction term, the presence of the *PPARG* Pro12 resulted in a 64% risk of prevalent type 2 diabetes mellitus. Furthermore, the *PPARG* Pro12 was associated with increased levels of 2-hour post-OGTT insulin. Overall, our findings convincingly demonstrate that *PPARG*-

IRS1 interactions, *PPARG* Pro12 and the susceptibility to environmental factors might modulate the relationship between IR and T2DM in this population.

The gene-gene interaction between *IRS1*, Gly972Arg and *PPARG*, Pro12Ala is of interest because the two polymorphisms exert opposite effects on T2DM predisposition. The Gly972Arg is the most common polymorphism in the *IRS1* gene and individuals carrying the Gly972Arg have a 25% increased risk for developing type 2 diabetes mellitus (Jellema et al., 2003). On the other hand, the *PPARG* Pro12Ala has been associated with a reduced risk of T2DM and insulin resistance (Buzzetti et al., 2004; Ludovico et al., 2007; Gouda et al., 2010; Trombetta et al., 2013). As such, the polymorphisms of the *IRS1* and *PPARG* genes have been shown to interact and elevate insulin sensitivity. This was evident in a study done by Stumvoll et al. (2002), in which the authors demonstrated that IS was significantly greater in subjects with X/Ala (PPAR γ 2) + X/Arg (*IRS1* 972) than in subjects with Pro/Pro (PPAR γ 2) + X/Arg (*IRS1*), while no differences were observed in X/Ala (PPAR γ 2) + Gly/Gly (*IRS1* 972) and Pro/Pro (PPAR γ 2) + Gly/Gly (*IRS1* 972) carriers (Stumvoll et al., 2002). Similarly, the interaction between the two polymorphisms has been associated with higher adiponectin levels, and the greatest increase was found in subjects who were homozygous for both *PPARG* alanine (Ala12Ala) and *IRS1* glycine (Gly972Gly) (Mousavinasab et al., 2005). Adiponectin is secreted by the adipose tissue and is inversely associated with obesity, IR, T2DM and cardiovascular diseases (Weyer et al., 2001; Von Eynatten et al., 2006). All these reports, including the present study, confirm the combined effect of the two SNPs on IR and type 2 diabetes mellitus.

Several epidemiological studies have demonstrated that *PPARG* Pro12Ala is associated with IS and type 2 diabetes mellitus (Buzzetti et al., 2004; Ludovico et al., 2007; Gouda et al., 2010; Trombetta et al., 2013). In the Human Genome Epidemiology (HuGE) meta-analysis involving 32 849 T2DM cases and 47 456 controls, the Pro12Ala was associated with a 14% lower risk for developing type 2 diabetes mellitus (Gouda et al., 2010). However, other investigations have failed to demonstrate an association between Pro12Ala and IS using the gold standard method for assessing IR/S, the euglycemic hyperinsulinemic clamp (Hasstedt et al., 2001; Stefan et al., 2001). The differences between studies have been attributed to BMI and ethnic differences (Ludovico et al., 2007; Gouda et al., 2010). The frequency of the 12Ala was reported to be higher in Caucasians than in Asian populations (Gouda et al., 2010) but conferred a significantly greater protection against T2DM among Asians as opposed to Caucasians (35% vs. 15%), as demonstrated by Ludovico et al. (2007). However, when adjustments were made for BMI, the differences were no longer significant (Ludovico et al., 2007).

In the present study, the 10,4% frequency of Pro12Ala polymorphism is comparable to that found in Caucasians, and the Pro12 was strongly associated with increased 2-hour post-OGTT insulin levels in non-diabetic subjects. These results further add to the growing body of evidence regarding the association of *PPARG* Pro12Ala with IR and subsequent type 2 diabetes mellitus. Herein, a heterogeneous population was investigated, comprising 32-43% Khoisan, 20-36% Bantu-speaking African, 21-28% European and 9-11% Asian ancestry (De Wit et al., 2010). The present findings require replication in a larger study involving other homogenous populations before the results can be considered established in Africa.

The strengths of the present study include the use of both fasting and OGTT derived indices for assessing T2DM and insulin resistance. The OGTT derived indices are found to have superior predictive power compared to simple fasting indices of IR because post-load glucose-insulin interaction is taken into account (Hancox & Landhuis, 2011). Furthermore, two independent laboratories to genotype the study population were employed. The main limitation of this study is the statistical power of the study, which was limited by the small sample size, and the examination of the gene-gene interaction effects reduced the sample further. Nevertheless, the results of this study provide the first preliminary evidence for genetic predisposition to IR and subsequent T2DM in an African population with a high prevalence of type 2 diabetes mellitus. In conclusion, the *PPARG* Pro12 is associated with IR, and this polymorphism interacts with an additional unfavourable genetic polymorphism, *IRS1* Gly972Arg, to increase the risk of T2DM in a mixed-ancestry population of South Africa.

Table 3.1 General characteristics of the overall population and by diabetes status

Variable	No diabetes	Diabetes	p-value	Overall
Number	575	212		787
Gender, male n (%)	131 (23)	45 (21)	0,642	176
Hypertension, n (%)	306 (53)	138 (65)	0,003	444
<u>Mean(SD)</u>				
Age (years)	51,3 (15,5)	59,3 (13,4)	<0,0001	53,5 (15,4)
Systolic blood pressure (mmHg)	123 (19)	131 (23)	<0,0001	124 (21)
Diastolic blood pressure (mmHg)	75 (12)	78 (15)	0,035	76 (13)
Body mass index (kg/m ²)	29,1 (7,1)	31,7 (7,2)	<0,0001	29,8 (7,2)
Waist circumference (cm)	95 (15)	102 (14)	<0,0001	97 (16)
Hip circumference (cm)	109 (14)	111 (15)	0,028	109 (14)
Waist-to-hip ratio (cm)	0,87 (0,10)	0,92 (0,09)	<0,0001	0,88 (0,10)
HbA1c (%)	5,7 (0,4)	7,8 (2,1)	<0,0001	6,3 (1,5)
HbA1c (Mmol/mol)	39 (4,4)	62 (23)	<0,0001	45 (16,4)
FBG (Mmol/L)	5,1 (0,7)	9,8 (4,4)	<0,0001	6,4 (3,1)
2h glucose (Mmol/L)	6,4 (1,6)	13,4 (5,3)	<0,0001	7,3 (3,5)
eGFR (mL/min)	76,0 (21,1)	71,2 (25,2)	0,015	74,7 (22,4)
Triglycerides (Mmol/L)	1,4 (0,9)	1,7 (0,9)	<0,0001	1,5 (0,9)
HDL cholesterol (Mmol/L)	1,3 (0,4)	1,2 (0,3)	0,0001	1,3 (0,4)
LDL cholesterol (Mmol/L)	3,6 (1,0)	3,7 (1,1)	0,191	3,6 (1,0)
Total cholesterol (Mmol/L)	5,5 (1,2)	5,7 (1,3)	0,070	5,6 (1,2)
<u>Median(25th-75th percentiles)</u>				
GGT	26 [18-39]	31 [23-39]	<0,0001	27 [19-42]
CRP	3,4 [0,8-8,4]	5,2 [1,9-10,8]	<0,0001	4,0 [1,1-9,4]
Insulin (Mmol/L)	6,9 [3,3-12,5]	9,2 [3,7-16,6]	0,0009	7,5 [3,3-13,5]
2h insulin (Mmol/L)	35,3 [19,2-64,5]	58,9 [22,1-115,2]	0,0009	36,8 [19,5-72,7]
Glucose/Insulin ratio	0,72 [0,42-1,51]	0,88 [0,50-2,30]	0,016	0,75 [0,43-1,68]
HOMA-IR	1,6 [0,7-2,9]	3,5 [1,5-6,7]	<0,0001	1,9 [0,8-3,7]
HOMA-β %	90,0 [41,1-160,0]	40,7 [12,4-77,8]	<0,0001	71,2 [28,6-44,9]
QUICKI	0,15 [0,14-0,18]	0,14 [0,13-0,15]	<0,0001	0,15 [0,14-0,17]
FIRI	1,4 [0,6-2,6]	3,1 [1,3-6,0]	<0,0001	1,8 [0,7-3,3]
1/HOMA-IR	0,64 [0,34-1,49]	0,29 [0,15-0,66]	<0,0001	0,54 [0,27-1,26]

Abbreviations: CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; FIRI, fasting insulin resistance index; GGT, γ -glutamyltransferase; HbA1c, glycated haemoglobin; HDL, high-density lipoproteins; HOMA- β %, homeostasis model assessment of functional beta-cells; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoproteins; QUICKI, quantitative insulin sensitivity check index; SD, standard deviation

Table 3.2 Genotype distributions, minor allele frequencies and unadjusted p-values for comparing genotype distribution according to diabetes status, including additive allelic effects between diabetes groups

	No diabetes	Diabetes	p-value	Overall
N	575	212		787
<i>IRS1</i>				
G/G, n (%)	526 (91,5)	199 (93,9)	0,485	725 (92,1)
G/A, n (%)	48 (8,3)	13 (6,1)		61 (7,7)
A/A, n (%)	1 (0,2)	0 (0)		1 (0,1)
A, n (%)	97 (8,4)	26 (6,1)	0,131	123 (7,8)
HWE (p-value)	>0,999	>0,999		>0,999
<i>PPARG</i>				
C/C, n (%)	521 (90,6)	183 (86,3)	0,161	704 (89,4)
C/G, n (%)	53 (9,2)	29 (13,7)		82 (10,4)
G/G, n (%)	1 (0,2)	0 (0)		1 (0,1)
G, n (%)	107 (9,3)	58 (13,7)	0,012	165 (10,5)
HWE (p-value)	>0,999	0,605		0,719

HWE, Hardy-Weinberg Equilibrium (HWE p-values are from exact tests).

Abbreviations: ***IRS1***, insulin substrate receptor 1; ***PPARG***, peroxisome proliferator-activated receptor gamma

Table 3.3 Generalised linear regression models* showing the effects of genes on markers of insulin resistance/sensitivity

Allele	Phenotype	Overall		No diabetes		Diabetes		p interaction
		Effects size (95%CI)	p	Effects size (95%CI)	p	Effects size (95%CI)	p	
IRS1 A	FBG	-0,06 (-0,66 to 0,54)	0,839	0,09 (-0,11 to 0,30)	0,372	-0,53 (-2,96 to 1,90)	0,668	0,330
	2h glucose	0,32 (-0,36 to 0,99)	0,355	0,08 (-0,36 to 0,51)	0,723	2,33 (-2,09 to 6,75)	0,304	0,038
	HbA1c	0,03 (-0,26 to 0,33)	0,821	0,02 (-0,10 to 0,14)	0,746	0,13 (-1,04 to 1,31)	0,823	0,853
	Fasting insulin	-1,47 (-6,09 to 3,15)	0,533	-1,02 (-3,76 to 1,71)	0,463	-3,27 (-20,75 to 14,21)	0,714	0,690
	2h insulin	1,71 (-15,6 to 19,0)	0,846	1,62 (-15,50 to 18,75)	0,853	2,36 (-63,13 to 67,86)	0,944	0,944
	Glucose/insulin	-0,08 (-2,68 to 2,52)	0,952	-0,14 (-2,26 to 1,97)	0,894	0,30 (-8,40 to 9,00)	0,946	0,896
	HOMA-IR	-0,58 (-2,52 to 1,36)	0,558	-0,22 (-0,87 to 0,42)	0,498	-2,03 (-9,96 to 5,91)	0,617	0,448
	QUICKI	0,001 (-0,013 to 0,014)	0,905	0,002 (-0,015 to 0,018)	0,857	-0,001 (-0,025 to 0,022)	0,903	0,877
	FIRI	-0,52 (-2,26 to 1,22)	0,558	-0,20 (-0,78 to 0,38)	0,480	-1,82 (-8,97 to 5,31)	0,617	0,448
PPARG C	FBG	-0,02 (-0,55 to 0,52)	0,948	-0,06 (-0,26 to 0,13)	0,528	0,003 (-1,71 to 1,72)	0,995	0,970
	2h glucose	-0,47 (-1,10 to 0,16)	0,148	-0,02 (-0,44 to 0,41)	0,933	-2,46 (-5,68 to 0,76)	0,137	0,002
	HbA1c	0,004 (-0,260 to 0,278)	0,976	0,07 (-0,04 to 0,19)	0,216	-0,18 (1,02 to 0,65)	0,663	0,362
	Fasting insulin	-2,07 (-6,17 to 2,02)	0,327	-0,56 (-3,21 to 2,09)	0,617	-5,72 (-18,00 to 6,57)	0,363	0,283
	2h insulin	23,2 (5,8 to 40,7)	0,009	34,0 (15,9 to 52,2)	0,0003	-14,4 (-64,7 to 35,8)	0,575	0,017
	Glucose/insulin	-1,08 (-3,4 to 1,2)	0,362	-0,21 (-2,26 to 1,83)	0,840	-2,62 (-8,82 to 3,37)	0,407	0,307
	HOMA-IR	-0,70 (-2,4 to 1,0)	0,421	-0,12 (0,74 to 0,51)	0,714	-2,31 (-7,89 to 3,27)	0,417	0,299
	QUICKI	-0,003 (-0,014 to 0,009)	0,675	0,0001 (-0,015 to 0,016)	0,924	-0,007 (-0,024 to 0,009)	0,377	0,481
	FIRI	-0,63 (-2,18 to 0,91)	0,421	-0,10 (-0,67 to 0,46)	0,714	-2,08 (-7,10 to 2,94)	0,417	0,299

* Models are adjusted for age, sex and diabetes.

Abbreviations: FBG, Fasting blood glucose; FIRI, fasting insulin resistance index; HbA1c, glycated haemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; **IRS1**, insulin substrate receptor 1; **PPARG**, peroxisome proliferator-activated receptor gamma; QUICKI, quantitative insulin sensitivity check index

Table 3.4 Odds ratio and 95% confidence intervals from logistic regression for the prediction of diabetes

Allele	Covariates	OR (95% CI)	p-value
<i>IRS1 A</i>	Gene alone	0,69 (0,36-1,25)	0,250
	sex, age	0,67 (0,34-1,24)	0,228
	sex, age, insulin	0,70 (0,35-1,31)	0,290
	sex, age, 2h insulin	0,76 (0,28-1,76)	0,562
	sex, age, HOMA-IR	0,72 (0,35-1,39)	0,347
	sex, age, QUICKI	0,70 (0,35-1,31)	0,285
	sex, age, FIRI	0,72 (0,35-1,39)	0,347
	sex, age, glucose/insulin	0,67 (0,34-1,27)	0,224
	<i>PPARG</i>	0,69 (0,36-1,25)	0,248
	<i>PPARG, IRS1*PPARG</i>	0,85 (0,43-1,57)	0,613
<i>PPARG C</i>	Gene alone	1,48 (0,91-2,36)	0,104
	sex, age	1,40 (0,85-2,28)	0,176
	sex, age, insulin	1,49 (0,90-2,45)	0,116
	sex, age, 2h insulin	1,24 (0,56-2,53)	0,571
	sex, age, HOMA-IR	1,51 (0,88-2,56)	0,131
	sex, age, QUICKI	1,40 (0,83-2,32)	0,198
	sex, age, FIRI	1,51 (0,88-2,56)	0,131
	sex, age, glucose/insulin	1,41 (0,85-2,51)	0,174
	<i>IRS1</i>	1,48 (0,91-2,37)	0,103
	<i>IRS1, IRS1*PPARG</i>	1,64 (1,00-2,64)	0,046

*Interaction term

Abbreviations: FIRI, fasting insulin resistance index; HOMA-IR, homeostasis model assessment of insulin resistance; *IRS1*, insulin substrate receptor 1; *PPARG*, peroxisome proliferator-activated receptor gamma; QUICKI, quantitative insulin-sensitivity check index

CHAPTER 4

LOW FREQUENCY MUTATIONS OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (*PPARG*) IN THE MIXED-ANCESTRY POPULATION FROM SOUTH AFRICA

4.1 BACKGROUND

Peroxisome proliferator-activated receptor gamma (*PPARG*) is one of the three PPAR isotypes, which include PPAR alpha and PPAR beta. The *PPARG* is mainly expressed in adipose tissue, with PPAR alpha mostly in brown adipose tissue and liver and PPAR beta being found in many tissues but mostly expressed in the gut, kidney and heart (Desvergne & Wahli, 1999; Wang et al., 1999). The expression of peroxisome proliferator-activated receptors (PPARs) occurs when hetero-dimers are formed with the retinoid X receptor (RXR) and bind to specific PPAR responsive elements of DNA to promote transcription. The PPAR:RXR complex is activated via ligand binding to the PPARs, resulting in the release of co-repressors bound to the receptor and recruitment of co-activators to initiate transcription of target genes (Mukherjee et al., 2000; Deeb et al., 1998; Pascual et al., 2005). For example, antidiabetic drugs of the thiazolidinedione family are believed to target the transcription factor *PPARG* to improve IS in T2DM and induce GLUT4 mRNA expression in fat and muscle (Armoni et al., 2003; Cariou et al., 2012; Yau et al., 2013).

In several studies, naturally occurring *PPARG* polymorphisms have been described. The two common variants of the *PPARG* gene, Pro12Ala and His449His have been associated with metabolic states of obesity, IR, T2DM and metabolic syndrome (Ding et al., 2012). The His449His polymorphism (also referred to as C161T, C1431T or CAC478CAT, His447His) is a silent mutation at exon 6 and is considered a better predictor of fasting insulin levels and IR than Pro12Ala (Moffet et al., 2005). In an earlier study, the presence of *PPARG* Pro12 was found to produce a 64% risk of prevalent T2DM, but an association with markers of IR was not observed (Vergotine et al., 2014). Therefore, in the present study, the association between His449His and other low frequency *PPARG* polymorphisms with cardio-metabolic traits was investigated.

4.2 MATERIALS AND METHODS

4.2.1 Baseline evaluations

This investigation is based on the Bellville South Africa cohort from Cape Town, South Africa. The study setting, survey design and procedures have been described in detail in previous research (Erasmus et al., 2012; Matsha et al., 2013; Zemlin et al., 2011) as well as in chapter 2. It has received study approval from the Research Ethics Committee of Stellenbosch University (HREC

Reference number: N09/05/146) and CPUT, Faculty of Health and Wellness Sciences Ethics Committee (Reference Numbers: CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants received a standardised interview and physical examination during which blood pressure was measured according to the World Health Organization (WHO) guidelines (Chalmers et al., 1999) in a sitting position using a semi-automated digital blood pressure monitor (Rossmax PA, USA) on the right arm. Anthropometric measurements were performed three times and their averages used for analysis: weight (kg), height (cm), waist (cm) and hip (cm) circumferences. Participants with no history of doctor-diagnosed diabetes mellitus underwent a 75 g OGTT as recommended by WHO (Alberti & Zimmet, 1998). The following biochemical parameters were determined on the Cobas 6000 Clinical Chemistry instrument (Roche Diagnostics, Germany): fasting plasma glucose, insulin, creatinine, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), triglycerides (TG), C-reactive protein (CRP), γ -glutamyltransferase (GGT) and glycated haemoglobin (HbA1c), certified by the National Glycohaemoglobin Standardisation Programme (NGSP). Low-density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (Friedewald et al., 1972).

4.2.2 Single Nucleotide Polymorphisms genotyping

Genomic DNA was extracted from whole blood samples collected in EDTA tubes. Single nucleotide polymorphisms in the *PPARG*, Pro115Gln (rs1800571,G>T), Val290Met (rs72551362,G>A), Phe388Leu (rs72551363,T>A), Arg397Cys (rs72551364,C>T) and His449His (rs3856806,C>T) were genotyped using the high-throughput real-time polymerase chain reaction. This was carried out in two independent laboratories on the ABI Prism 7900HT platform (Applied Biosystems, USA) and a BioRad Optica (BioRad, USA), using the Taqman genotyping assay (Applied Biosystems, USA). Conventional polymerase chain reaction, followed by direct DNA sequencing, was performed for the analytical validation of high-throughput genotyping.

4.2.3 Definitions and calculations

The BMI was calculated as weight per square metre (kg/m^2) and the waist-hip ratio (WHR) as waist/hip circumferences (cm). The T2DM status was based on a history of doctor diagnosis, a fasting plasma glucose $\geq 7,0\text{mmol/L}$ and/or a 2-hour post-OGTT plasma glucose $\geq 11,1\text{mmol/L}$. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: $\text{HOMA-IR} = [\text{fasting insulin concentration (mIU/L)} \times \text{fasting plasma glucose (mmol/L)}] / 22,5$; while homeostasis model assessment of functional beta-cells (HOMA- $\beta\%$) were estimated using the formula: $20 \times \text{fasting insulin (}\mu\text{IU/mL)} / \text{fasting glucose (mmol/mL)} - 3,5$. The fasting insulin resistance index (FIRI) was calculated with the formula: $[\text{fasting insulin (}\mu\text{U/mL)} \times$

fasting glucose (mM)]/25 and the quantitative insulin sensitivity check index (QUICKI) as: $1/[\log(\text{fasting insulin } (\mu\text{U/mL}) \times \log(\text{fasting glucose (mg/dL)})]$. The glomerular filtration rate (eGFR) was estimated by the 4-variable MDRD equation (Levey et al., 1999; Levey et al., 2006), applicable to standardised serum creatinine values.

4.3 STATISTICAL ANALYSIS

The general characteristics of the study group are summarised as count and percentage for dichotomous traits, mean and standard deviation (SD) or median and 25th-75th percentiles for quantitative traits. Where necessary, traits were log-transformed to approximate normality prior to analysis. The SNPs were tested for departure from the HWE expectation via a chi square goodness of fit test. Linear regression models were used for the analysis of quantitative traits and logistic regression models for dichotomous traits, always assuming additive models for the single nucleotide polymorphisms. Using linear and logistic models enabled the adjustment of all analyses for known confounders, as specified in the results. The additive allelic association of each SNP with each trait was investigated, overall and according to the T2DM status, and tested for heterogeneity by adding the interaction term of T2DM and each SNP to a model that contained the main effects of T2DM and the relevant single nucleotide polymorphism. Results corresponding to the p-values below 5% are described as significant, with borderline defined statistically as $p > 0,005$ but $p < 0,1$. There were no adjustments for multiple testing. All analyses used the statistical package R (version 3.0.0 {2013-04-03}, the R Foundation for statistical computing, Vienna, Austria).

4.4 RESULTS

Of the 946 participants in the survey, 941 consented to genetic studies. Among the latter, 121 were excluded due to missing data on the genetic variables. Therefore, 820 had valid data for the current analyses. The clinical characteristics of participants overall and according to diabetes status are summarised in Table 4.1 (see p 58), indicating that 222 (27%) participants had type 2 diabetes mellitus. As expected, the distribution of the level of IR/S indicators was significantly different in the two groups (all $p < 0,0001$, except for the glucose/insulin ratio ($p = 0,0304$). Furthermore, compared with the non-diabetic participants, those with diabetes were significantly older (58,9 years vs. 51,0 years, $p < 0,0001$) and had higher levels of adipometric variables (all $p \leq 0,014$), systolic blood pressure ($p < 0,0001$), TG ($p < 0,0001$), GGT and CRP (both $p < 0,0001$), whilst eGFR ($p = 0,029$) and HDL cholesterol ($p = 0,0002$) were significantly lower.

No mutations were found for the following SNPs of *PPARG* Pro115Gln, Val290Met, Phe388Leu and Arg397Cys. The *PPARG*, His449His variant was in HWE ($p > 0,05$). The genotype and allele distribution by diabetes status is summarised in Table 4.2 (see p 59). The frequency of the *PPARG* (rs3856806), C/T genotype was 23,8% in the overall population group and the genotype

distributions did not differ significantly between the non-diabetes and diabetes groups. Overall, the T allele frequency was 14%, however, although not significant ($p=0,128$) the T allele did occur more in the non-diabetes (14,8%) than in the diabetes (11,7%) participants.

In the generalised linear regression analyses adjusted for age, sex and diabetes (Table 4.3: p 60), the *PPARG* allele T was not associated with any markers of glycaemia, IR/S overall and by diabetes status. There was a borderline statistical interaction in the effect by diabetes status for the association with fasting blood glucose (interaction $p=0,092$), HOMA-IR and FIRI indexes (both $p=0,076$). Furthermore, the effects always appeared to be sizeable and negative (although non-significant) among the diabetes and almost null in the non-diabetes participants.

In the logistic regression models (Table 4.4: p 61), the *PPARG* allele T was associated with an odd ratio of 0,76 (95% confidence interval (CI): 0,55–1,06) for prevalent diabetes. The effect generally remained within the same range after adjustment for age and sex, with or without further adjustment for the various indices of insulin resistance/sensitivity. However, in the model containing sex, age and the 2-hour insulin, the *PPARG* allele T was significantly associated with a lower prevalent diabetes risk, demonstrating an odd ratio of 0,56 (95% CI: 0,31–0,95).

4.5 DISCUSSION

The present study reports on five *PPARG* SNPs: rs1800571, rs72551362, rs72551363, rs72551364 and rs3856806. Only one of these *PPARG* variants, His449His (rs3856806) was found in the mixed ancestry population and demonstrated an association with diabetes. The T allele frequencies were about 14% and, in an additive genetic model we observed that the presence of the T allele significantly reduced the risk of diabetes by 44%. Furthermore, the T allele was associated with reduced fasting blood glucose levels, HOMA-IR and FIRI indices. However, these associations were borderline and did not reach statistical significance.

The nuclear transcription receptor, *PPARG*, acts as a regulator of adipocyte differentiation, pancreatic beta cell function, lipid and glucose metabolism (Spiegelman, 1997; Rosen et al., 2003; Hevener et al., 2003). Despite its biological plausibility, previous reports that have studied the association with obesity (Valve et al., 1999; Meirhaeghe et al., 1998; Dongxia et al., 2008), IR (Moffet et al., 2005; Dongxia et al., 2008), T2DM (Tai et al., 2004; Vergotine et al., 2014) and MetS (Youssef et al., 2014) have been inconsistent. Some studies have reported that carriers of the T allele of His449His (rs3856806) have an increased obesity risk (Valve et al., 1999) and a poor lipid profile (Gu et al., 2013; Yilmaz-Aydogan et al., 2013; Rhee et al., 2006), as well as an increased risk of MetS (Youssef et al., 2013) and coronary heart diseases (CHD) (Ding et al., 2012). On the other hand, others have failed to demonstrate an association between *PPARG* SNPs,

including C161T with CHD susceptibility (Xu et al., 2013). Additional research has shown an association with increased IS (Moffet et al., 2005; Dongxia et al., 2008), decreased CHD, BMI and diabetes risk (Dongxia et al., 2008; Tai et al., 2004; Rhee et al., 2006; Liu et al., 2007) in subjects harbouring the T allele.

In this study, the presence of the T allele was associated with reducing the risk of prevalent diabetes and levels of the IR indices. The inconsistencies observed in these studies include variations in the ethnic distribution of the *PPARG* polymorphisms, power of the studies, environmental-gene, gene-gene interaction as well as different levels of adjustment for the potential confounding factors across studies. For example, in this study, the T allele frequency of the His449His variant was 14%, which is similar to that reported in Australian Caucasians at 16,3% (Wang et al., 1999) and in Tunisians at 18,3% (Youssef et al., 2014), whilst in Chinese subjects a frequency of 29,2% has been reported (Luo et al., 2013). Furthermore, two recent studies have shown that gene-gene interaction analysis is superior to single *PPARG* polymorphisms, particularly regarding low frequency SNPs, for the quantification of effects with cardiometabolic traits (Luo et al., 2013; Chan et al., 2013). Luo et al. (2013) investigated the association of ten *PPARG* SNPs with obesity and showed that only two SNPs, rs2016520 and rs10865170, were associated with a lower obesity risk. However, in generalised multifactor dimensionality reduction analysis to assess the effect of interaction among the ten SNPs, an additional SNP, rs9794, was identified, which showed a potential gene-gene interaction with rs2016520 and rs10865170 (Luo et al., 2013).

The low frequency *PPARG* SNPs, rs1800571, rs72551362, rs72551363, rs72551364, were also genotyped, but no genetic variations were observed. The rs1800571, Pro115Gln gain-of-function mutation has only been found in five German morbidly obese subjects since its initial identification by Ristow et al. (1998), where three of the four obese subjects with the genetic variant also had type 2 diabetes mellitus. In another German population study, another carrier of the mutant with severe IR was identified (Bluher & Paschke, 2003). However, other studies involving German, Danish and American populations failed to find this mutation (Hamann et al., 1999; Evans et al., 2000; Ek et al., 1999; Shuldiner et al., 2000; Hegele et al., 2002). Similarly, there has not been more than one individual in which either rs72551362, rs72551363 or rs72551364 has been found (Hegele et al., 2002; Barosso et al., 1999; Agarwal & Garg, 2002).

The rare nature of these mutations suggests that standard population-based type studies are likely not the suitable design to investigate them, considering the requirements for very large sample size. Rather targeting specific segments of the population such as people with morbid obesity for screening may increase the likelihood of uncovering some with the mutation. It is also possible that

mutations other than those reported so far could be present in other populations, indicating the need for genome-wide scanning studies in different setting to capture additional mutations.

The present study has limitations and these include the investigation of only five SNPs and the small sample size. However, the participants in this study were well characterised with both fasting and oral glucose tolerance test derived indices, which were used for the assessment of T2DM and insulin resistance. In conclusion, this study confirmed the almost zero occurrences of rare *PPARG* SNPs and has shown that one of the common SNPs, His449His may be protective against type 2 diabetes mellitus. Future larger studies with more SNPs involving populations from Africa need further exploration.

Table 4.1 General characteristics of the overall population and by diabetes status

Variable	No diabetes	Diabetes	p-value	Overall
Number	598	222		820
Gender, male n (%)	130 (22)	48 (22)	0,9474	178
Hypertension, n (%)	314 (53)	133 (60)	0,05859	447
<u>Mean(SD)</u>				
Age (years)	51,0 (15,1)	58,9 (13,4)	<0,0001	53,2 (15,1)
Systolic blood pressure (mmHg)	122 (19)	130 (23)	<0,0001	124 (21)
Diastolic blood pressure (mmHg)	75 (12)	77 (14)	0,0506	76 (13)
Body mass index (kg/m ²)	29,2 (7,0)	31,8 (7,1)	<0,0001	29,9 (7,1)
Waist circumference (cm)	95 (15)	102 (14)	<0,0001	97 (15)
Hip circumference (cm)	109 (14)	112 (15)	0,014	109 (14)
Waist-to-hip ratio (cm)	0,87 (0,10)	0,91 (0,10)	<0,0001	0,88 (0,09)
HbA1c (%)	5,7 (0,4)	7,8 (2,1)	<0,0001	6,3 (1,4)
FBG (Mmol/L)	5,1 (0,7)	9,8 (4,5)	<0,0001	6,4 (3,2)
2h glucose (Mmol/L)	6,4 (1,6)	13,4 (5,3)	<0,0001	7,5 (3,6)
eGFR (mL/min)	76,2 (21,2)	72,0 (25,2)	0,029	75,0 (22,4)
Triglycerides (Mmol/L)	1,4 (0,9)	1,7 (0,9)	<0,0001	1,5 (0,9)
HDL cholesterol (Mmol/L)	1,3 (0,4)	1,2 (0,3)	0,0002	1,3 (0,4)
LDL cholesterol (Mmol/L)	3,6 (1,0)	3,7 (1,1)	0,179	3,6 (1,0)
Total cholesterol (Mmol/L)	5,5 (1,2)	5,7 (1,3)	0,068	5,6 (1,2)
<u>Mean(25th -75th percentiles)</u>				
GGT	26 [17-39]	32 [23-49]	<0,0001	27 [19-42]
CRP (mg/L)	3,3 [0,9-8,3]	5,4 [2,2-10,9]	<0,0001	4,0 [1,2-9,4]
Insulin (Mmol/L)	7,2 [3,4-12,9]	10,2 [4,4-17,6]	0,0001	7,7 [3,5-14,1]
2h insulin (Mmol/L)	36,0 [19,3-65,7]	53,3[22,1-117,0]	0,0015	37,3 [19,5-72,7]
Glucose/insulin	0,71 [0,40-1,45]	0,82 [0,48-2,05]	0,0304	0,72 [0,41-1,58]
HOMA-IR	1,63 [0,69-3,00]	3,67 [1,71-7,29]	<0,0001	1,94 [0,86-3,82]
HOMA-β%	92,8 [43,5-165,1]	42,1 [13,6-83,4]	<0,0001	74,2 [32,2-149,2]
QUICKI	0,15 [0,14-0,18]	0,14 [0,12-0,15]	<0,0001	0,15 [0,14-0,17]
FIRI	1,46 [0,62-2,70]	3,31 [1,54-6,56]	<0,0001	1,74 [0,77-3,44]
1/HOMA-IR	0,61 [0,33-1,44]	0,27 [0,14-0,58]	<0,0001	0,52 [0,26-1,16]

Abbreviations: CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; FBG, Fasting blood glucose; FIRI, fasting insulin resistance index; GGT, γ-glutamyltransferase; HbA1c, glycated haemoglobin; HDL, high density lipoproteins; HOMA-β%, homeostasis model assessment of functional beta-cells; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low density lipoproteins; QUICKI, the quantitative insulin sensitivity check index; SD, standard deviation

Table 4.2 The genotype distributions, minor allele frequencies and unadjusted p-values for comparing genotype distribution according to diabetes status, including additive allelic effects between diabetes groups

	No diabetes	Diabetes	p-value	Overall
N	598	222		820
<u>PPARG (rs3856806)</u>				
C/C, n (%)	436 (72,9)	172 (77,5)	0,215	608 (74,1)
C/T, n (%)	147 (24,5)	48 (21,6)		195 (23,8)
T/T, n (%)	15 (2,5)	2 (0,9)		17 (2,1)
T, n (%)	177 (14,8)	52 (11,7)	0,128	229 (14,0)
HWE (p-value)	0,517	0,747		0,771

HWE, Hardy-Weinberg Equilibrium (HWE p-values are from exact tests).

Abbreviations: **PPARG**, peroxisome proliferator-activated receptor gamma

Table 4.3 Generalised linear regression models* showing the effects of genes on markers of insulin sensitivity /resistance

Allele	Phenotype	Overall		No diabetes		Diabetes		p interaction	
		Effects size (95%CI)	p	Effects size (95%CI)	p	Effects size (95%CI)	p		
<i>PPARG</i> (rs3856806)T	FBG	-0,140 (-0,480 to 0,199)	0,417	0,0002 (-0,112 to 0,112)	0,996	-0,606 (-1,934 to 0,722)	0,372	0,092	
	2h glucose	-0,114 (-0,494 to 0,266)	0,557	-0,098 (-0,341 to 0,144)	0,426	-0,147 (-2,826 to 2,533)	0,915	0,847	
	HbA1c	-0,018 (-0,179 to 0,143)	0,826	0,034 (-0,031 to 0,099)	0,303	-0,211 (-0,829 to 0,407)	0,504	0,164	
	Fasting insulin	-0,915 (-3,362 to 1,533)	0,464	0,022 (-1,420 to 1,463)	0,976	-4,516 (-13,448 to 4,416)	0,323	0,125	
	2h insulin	-1,716 (-11,818 to 8,386)	0,739	0,408 (-9,473 to 10,289)	0,936	-20,212 (-62,232 to 1,807)	0,348	0,190	
	Glucose/insulin	-0,488 (-1,905 to 0,929)	0,500	-0,083 (-1,258 to 1,091)	0,890	-1,743 (-6,271 to 2,785)	0,451	0,334	
	HOMA-IR	-0,437 (-1,481 to 0,608)	0,413	0,021 (-0,327 to 0,368)	0,907	-2,228 (-6,336 to 1,880)	0,289	0,076	
	QUICKI	0,001 (-0,006 to 0,009)	0,738	0,0008 (-0,008 to 0,010)	0,851	0,004 (-0,009 to 0,016)	0,564	0,752	
	FIRI	-0,393 (-1,333 to 0,547)	0,413	0,019 (-0,294 to 0,332)	0,907	-2,005 (-5,703 to 1,692)	0,289	0,076	

* Models are adjusted for age, sex and diabetes

Abbreviations: FBG, Fasting blood glucose; FIRI, fasting insulin resistance index; HbA1c, glycated haemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; *PPARG*, peroxisome proliferator-activated receptor gamma; QUICKI, quantitative insulin sensitivity check index

Table 4.4 Odds ratio and 95% confidence intervals from logistic regression for the prediction of diabetes

Allele	Covariates	OR (95% CI)	p
<i>PPARG</i> T	Gene alone	0,76 (0,55 to 1,06)	0,112
	sex, age	0,74 (0,53 to 1,03)	0,082
	sex, age, insulin	0,75 (0,53 to 1,05)	0,098
	sex, age, 2h insulin	0,56 (0,31 to 0,95)	0,042
	sex, age, HOMA-IR	0,77 (0,53 to 1,10)	0,157
	sex, age, QUICKI	0,75 (0,53 to 1,04)	0,094
	sex, age, FIRI	0,77 (0,53 to 1,10)	0,157
	sex, age, glucose/insulin	0,75 (0,53 to 1,04)	0,092

Abbreviations: FIRI, fasting insulin resistance index; HOMA-IR, homeostasis model assessment of insulin resistance; *PPARG*, peroxisome proliferator-activated receptor; QUICKI, quantitative insulin sensitivity check index

CHAPTER 5

INDICES OF INSULIN RESISTANCE AND MARKERS OF SUB-CLINICAL CARDIOVASCULAR DISEASE/ATHEROSCLEROSIS

5.1 BACKGROUND

Insulin resistance predisposes populations to several metabolic abnormalities such as dysglycaemia, dyslipidaemia, elevated blood pressure, a procoagulant state, inflammation and endothelial dysfunction. These are strongly associated with the development of diabetes and subsequently, cardiovascular diseases. As such, indices of IR have been developed using particular sampling protocols during the OGTT for the estimation of insulin sensitivity. This is an important metabolic normality parameter due to its potential role in the development of cardiometabolic alterations (Reaven, 2005; Bobbioni-Harsch et al., 2012). These surrogate markers were validated against the euglycaemic clamp technique (Matsuda & DeFronzo, 1999; Hanley et al., 2003) and are used for CVD risk stratification (Kannan & Chernoff, 2013).

However, these IR indices perform differently in different populations. For example, the homeostasis model of assessment of insulin resistance (HOMA-IR) is the most frequently used index to evaluate IR using measures derived from fasting states (Matthews et al., 1985). In a recent study, a significant positive correlation was reported between log HOMA-IR and the severity of coronary artery disease in diabetic patients (Srinivasan et al., 2013), thus highlighting the possible key role of IR in atherosclerosis. However, in a multi-ethnic study, the determination of HOMA-IR did not improve the detection of subclinical atherosclerosis (Bertoni et al., 2007). Another surrogate marker, the product of fasting triglycerides (TG) and the glucose (TyG) index, showed high sensitivity and specificity when compared to the clamp technique in identifying subjects with a decreased insulin sensitivity. This was also approved as an alternative assessment of IR to HOMA-IR when measures of insulin are not available (Guerrero-Romero et al., 2010). Although traditional vascular risk factors are important in the development of atherosclerosis, it is claimed that they only explain 50% of the risk of cardiovascular diseases (Bartels et al., 2012). Thus, more studies are investigating additional surrogate markers in the search for new therapies to reduce CVD mortality.

In previous studies, mixed-ancestry South Africans were reported to have the second highest prevalence of diabetes (Levitt et al., 1993), and in the mixed ancestry was identified as the major factor responsible for the increased CVD risk in the 30-year cardiovascular risk profile study (Matsha et al., 2012). The mortality from CVD is two-to-fourfold higher in those with diabetes (Gu

et al., 1998), which could explain why CVD is the second leading cause of death after HIV/AIDS in South Africans (Bradshaw et al., 2003). Furthermore, the study by Matsha et al. (2012) the distribution of traditional diabetes risk factors, such as obesity, is not appreciably different in mixed-ancestry South Africans with and without diabetes (Matsha et al., 2012). Therefore, this study investigated whether indices of IR can aid in the stratification of high-risk individuals by using carotid intima media thickness.

5.2 RESEARCH DESIGN AND METHODS

5.2.1 Study setting and population

The study setting, survey design and procedures have been described in detail in previous research (Erasmus et al., 2012; Matsha et al., 2013; Zemlin et al., 2011) as well as in chapter 2. Briefly, eligible participants were invited to take part in a community-based survey from January 2008 to March 2009 (Cohort 1) and January 2011 to November 2011 (Cohort 2). The study was approved by the Research Ethics Committee of Stellenbosch University (HREC Reference Number: N09/05/146) and the Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences Ethics Committee (Reference Numbers: CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed consent forms after all the procedures were fully explained in the language of their choice.

5.2.2 Clinical data

All consenting participants received a standardised interview and physical examination during which blood pressure was measured according to the World Health Organisation (WHO) guidelines (Chalmers et al., 1999) using a semi-automated digital blood pressure monitor (Rossmax PA, USA) on the right arm, with the participant in a sitting position. Other clinical measurements included the body weight, height, waist and hip circumferences. Weight (to the nearest 0,1 kg) was determined for a subject wearing light clothing and without shoes and socks, using a Sunbeam EB710 digital bathroom scale, which was calibrated and standardised using a weight of known mass. The waist circumference was measured using a non-elastic tape at the level of the narrowest part of the torso, as seen from the anterior view. The hip circumference was also measured using a non-elastic tape around the widest portion of the buttocks. All anthropometric measurements were performed three times and averaged then used for analysis. Participants with no history of doctor-diagnosed diabetes mellitus underwent a 75 g OGTT as recommended by WHO (Alberti & Zimmet, 1998).

5.2.3 Laboratory measurements

Blood samples were collected after an overnight fast and processed for further biochemical analyses. Plasma glucose was measured by the enzymatic hexokinase method (Cobas 6000, Roche Diagnostics, Germany) and glycated haemoglobin (HbA1c) by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics, Germany). This is a National Glycohaemoglobin Standardisation Programme (NGSP) certified method. Creatinine levels were measured using the standardised creatinine assay (Cobas 6000, Roche Diagnostics, Germany). Total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), triglycerides (TG) and γ -glutamyltransferase (GGT) were estimated by enzymatic colorimetric methods (Cobas 6000, Roche Diagnostics). Low density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (Friedewald et al., 1972). Insulin was determined by a microparticle enzyme immunoassay (AxSYM, Abbott). C-reactive protein was measured by a high-sensitivity CRP assay, based on the highly sensitive Near Infrared Particle Immunoassay rate methodology (Image® Immunochemistry System; Beckman Coulter), with a lower limit of detection of 0,2 mg/L.

5.2.4 Definitions and calculations

Body mass index (BMI) was calculated as weight per square metre (kg/m^2) and the waist-hip-ratio (WHR) as waist/hip circumferences (cm). The glomerular filtration rate was estimated by the 4-variable MDRD equation (Levey et al., 1999; Levey et al., 2006) applicable to the standardised serum creatinine values. Type 2 diabetes mellitus status was based on a history of doctor-diagnosis, a fasting plasma glucose $\geq 7,0$ Mmol/L and/or a 2-hour post-OGTT plasma glucose $\geq 11,1$ Mmol/L.

5.2.5 Surrogate measures of insulin resistance

The HOMA-IR was calculated according to the formula: $\text{HOMA-IR} = [\text{fasting insulin concentration (mIU/L)} \times \text{fasting plasma glucose (Mmol/L)}] / 22,5$; while homeostasis assessment of functional beta-cells ($\text{HOMA-}\beta\%$) were estimated using the formula: $20 \times \text{fasting insulin (}\mu\text{IU/mL)} / \text{fasting glucose (Mmol/mL)} - 3,5$. The fasting insulin resistance index (FIRI) was calculated with the formula: $[\text{fasting insulin (}\mu\text{U/mL)} \times \text{fasting glucose (mM)}] / 25$ and the quantitative insulin sensitivity check index (QUICKI) as: $1 / [\log (\text{fasting insulin (}\mu\text{U/mL)}) \times \log (\text{fasting glucose (mg/dL)})]$.

5.2.6 Measurement of carotid intima media thickness

Two qualified sonographers measured the CIMT in longitudinal section at the far wall of the distal common carotid arteries, 2 cm from the bifurcation, at three consecutive end-points, 5 mm to 10 mm apart. The CIMT was reported in millimetres and used as a measure of subclinical

CVD/atherosclerosis. The ultrasound examination was performed according to the ALARA principle to avoid biological effects that may be associated with ultrasound. The exposure output of the machine was kept to a low value with the thermal index <1 , which still gave an accurate diagnosis (Kremkau, 2006). The mean of six readings (three from each side) was calculated for each participant using a portable B-mode and spectral Doppler ultrasound scanner equipped with cardiovascular imaging software.

5.3 STATISTICAL ANALYSES

Data are presented as mean (standard deviation, SD) or median [25th-75th percentiles] for continuous variables and as count and percentage for categorical variables. For group (sex, diabetes status) comparisons, the chi square test and student t-test were used. Continuous associations between CIMT and the indices were assessed graphically with the use of a correlation matrix, before and after applying the Box-Cox power transformations to improve the shape of the associations. This was followed by the ‘Covariance Estimation for Multivariate t Distribution’ method to derive the correlation coefficients, while minimising the potential effects of outliers. The Steiger t-test was used to compare the correlation coefficients among the indices. Regression coefficients to indicate the size of the association of each of the indices with the CIMT measurements were derived from robust multiple linear regression models that included each of the four variables of interest: age, sex, BMI and diabetes status. Analyses were carried out using R statistical software (version 3.0.0 {03-04-2013}, the R Foundation for Statistical Computing, Vienna, Austria). The significance level was set at 0,05.

5.4 RESULTS

General characteristics of participants

Of the 515 participants included in this analysis, 137 (26,6%) were men and 150 (29,1%) had diabetes. The BMI ($p=0,002$), waist circumference ($p<0,0001$), waist-to-hip ratio ($p=0,0009$), systolic blood pressure ($p=0,0002$), TG ($p<0,0001$) and CIMT ($p<0,0001$) were significantly higher in the diabetic participants, whilst the HDL-c ($p=0,004$) was lower in the diabetic participants (Table 5.1: p 68). Significant differences were also apparent when comparing men and women with a number of variables including BMI, waist-hip ratio, TC, HDL-c, CRP, HbA1c, fasting blood insulin (FBI), IR markers, γ -glutamyltransferase, serum creatinine and carotid intima media thickness (Table 5.1: p 68).

Bivariate correlations

In the overall study sample, there was a weak positive correlation between CIMT and fasting glucose [$r=0,22$ (95% confidence intervals: 0,14 to 0,30)], HOMA-IR, FIRI and glucose/insulin ratio with a similar correlation coefficient of 0,09 (0,01 to 0,18), as seen in Table 5.2 (see p 69). In

participants with and without diabetes analysed separately, no significant correlation was observed between CIMT and markers of IR, with no differential effect between the two groups (all $p > 0,47$). The correlation coefficients were compared in the overall cohort and it was apparent that the correlation of CIMT with fasting blood glucose was stronger than that of CIMT with any of the other markers of insulin resistance (all $p < 0,02$).

The correlation of CIMT with fasting blood insulin was less than that with HOMA-IR or FIRI (both $p < 0,0001$) but not appreciably different from QUICKI ($p = 0,400$) or the glucose/insulin ratio ($p = 0,340$). Furthermore, the correlation of CIMT with QUICKI was significantly weaker than that with the glucose/insulin ratio ($p < 0,0001$) but only slightly weaker when compared to the correlation of CIMT with HOMA-IR and FIRI (both $p = 0,072$). See Table 5.2: p 69. The regression curves showing the linear correlations of CIMT with markers of IR are illustrated in Figure 5.1 (see p 70).

Multiple linear regression analyses

In a multiple robust linear regression model containing age, sex and BMI, the three variables were all significantly associated with CIMT (all $p < 0,003$) and explained 26,5% of variation in the CIMT values (Table 5.3: p 71). When the diabetes status was added to this model, the four variables in the model (basic model) were all significantly associated with CIMT (all $p < 0,023$) and together explained the 29.1% variation in the CIMT. In the presence of these four variables, fasting blood glucose ($\beta = 0,087$; $p = 0,042$) and glucose/insulin ratio ($\beta = 0,026$; $p = 0,026$) were positively associated with CIMT, while the association with other markers of IR did not reach statistical significance. Adding fasting blood glucose and glucose/insulin ratio to the basic model had a trivial effect on the overall performance of the model, with the highest achieved R^2 being only 29,7% (Table 5.3: p 71).

5.5 DISCUSSION

The findings of this study demonstrate that in a model containing traditional risk factors age, sex, adiposity and diabetes, the IR indices namely; FBG and glucose/insulin ratio were positively associated with carotid intima media thickness. However, the effect on the overall model performance by these IR indices were marginal, with the highest achieved R^2 of only 29,7%. The variance in CIMT explained by these IR indices did not differ much when compared to that of the traditional risk factors of 29,1%. Furthermore, CIMT as an independent predictor of CVD risk was found to be significantly higher in males and in participants with diabetes in the mixed-ancestry population.

A strong link between diabetes and CVD through the development of IR has been previously reported (Reviewed by Ikmal et al., 2013). Various epidemiological studies have shown an association between IR indices and carotid atherosclerosis, however, the results have been inconsistent (Bertoni et al., 2007; Kannan & Cherhoff, 2013; Jeppesen et al., 2007; Mehta et al., 2011; Sourij et al., 2008; Srinivasan et al., 2013; Vasques et al., 2011; Irace et al., 2013). Of the IR indices, HOMA-IR was found to be beneficial to identify patients with a higher CVD risk (Jeppesen et al., 2007; Sourij et al., 2008; Mehta et al., 2011; Srinivasan et al., 2013; Kannan & Cherhoff, 2013) with similar results reported for the TyG index (Vasques et al., 2011; Irace et al., 2013), fasting blood insulin, 2 hour postload insulin (Fontbonne et al., 1991) and the area under the plasma insulin response curve (Pyörälä et al., 1998). In contrast, a number of studies showed no evidence of association between IR indices such as HOMA-IR and TyG index for cardiovascular diseases (Bertoni et al., 2007; Vasques et al., 2011).

The positive and significant association of the IR indices, FBG and the glucose/insulin ratio with CIMT was found beyond the traditional risk factors in this study. Both these IR indices are derived from fasting blood levels representing the basal steady-state condition with respect to glycaemia, insulinaemia and hepatic glucose, reflecting primarily hepatic insulin resistance/sensitivity (Muniyappa et al., 2007). A stronger correlation of CIMT with FBG was observed in this study similar to the results obtained by Karbek et al. (2013). Fasting blood glucose has been previously associated with the risk of vascular disease in all concentrations, including at levels lower than 7 Mmol/L, the threshold for diabetes, however, data from studies are inconclusive (Coutinho et al., 1999; Lawes et al., 2004; Emerging Risk Factors Collaboration, et al., 2010; Emerging Risk Factors Collaboration et al., 2011). Changes in the glycaemic status can alter lipoprotein and lipid flux, which in turn can modulate IS and glucose disposal (Mazzone et al., 2008). The role of hyperglycaemia as a risk factor for CVD in T2DM has been linked to induced damage to the endothelium and arterial vasoconstriction, associated with vascular smooth muscle proliferation and increased intimal thickness (Massi-Benedetti & Federici, 1999). In two recent studies impaired glucose, which indirectly represents IR (Kim et al., 2013), and FBG levels were progressively associated with an increased risk of atherosclerosis cardiovascular diseases (Park et al., 2013).

Lastly, the measures of IR are weakly associated with CIMT, as there was only a small statistical difference when compared to subclinical disease linked to traditional risk factors for CVD, such as age, sex, adiposity and diabetes. In conclusion, the IR indices did not add independent value to the assessment of subclinical CVD risk in this mixed-ancestry population of South Africa.

Table 5.1 General characteristics of the overall population and by diabetes status

Variables	Overall	Sex		p	Diabetes		
		Men	Women		No	Yes	p
N	515	137	378		365	150	
Female, n (%)	378 (73,4)	0	378		263	115	0,282
Mean(SD)							
Age (years)	54,4 (13,3)	55,5 (13,6)	54,0 (13,1)	0,274	52,4 (13,4)	59,3 (11,6)	<0,0001
Body mass index (kg/m ²)	31,4 (8,1)	27,4 (7,0)	32,8 (8,0)	<0,0001	30,6 (7,4)	33,4 (9,4)	0,002
Waist circumference (cm)	96 (15)	94 (15)	97 (16)	0,096	95 (16)	101 (13)	<0,0001
Waist-to-hip ratio (cm)	0,89 (0,12)	0,93 (0,12)	0,87 (0,12)	<0,0001	0,88 (0,12)	0,92 (0,12)	0,0009
Systolic blood pressure (mmHg)	136 (26)	138 (24)	136 (27)	0,313	133 (23)	144 (30)	0,0002
Diastolic blood pressure (mmHg)	82 (14)	84 (15)	81 (13)	0,074	81 (14)	83 (14)	0,064
FBG (Mmol/L)	6,5 (3,1)	6,1 (2,4)	6,6 (3,2)	0,112	5,2 (0,7)	9,6 (4,1)	<0,0001
HbA1c (%)	6,6 (1,6)	6,3 (1,4)	6,7 (1,7)	0,007	5,9 (0,4)	8,3 (2,2)	<0,0001
Total cholesterol (Mmol/L)	5,5 (1,1)	5,6 (1,0)	5,5 (1,1)	0,660	5,5 (1,1)	5,6 (1,1)	0,610
HDL cholesterol (Mmol/L)	1,4 (0,4)	1,3 (0,5)	1,4 (0,4)	0,050	1,4 (0,4)	1,3 (0,4)	0,004
LDL cholesterol (Mmol/L)	3,4 (1,0)	3,4 (1,0)	3,5 (1,0)	0,776	3,5 (1,0)	3,4 (1,0)	0,633
Triglycerides (Mmol/L)	1,5 (0,9)	1,7 (1,1)	1,5 (0,8)	0,066	1,4 (0,8)	1,9 (1,1)	<0,0001
Creatinine (µM)	74,9 (25,2)	89,3 (34,6)	67,8 (18,3)	<0,0001	73,3 (18,5)	78,8 (36,6)	0,080
Median(25th -75th percentiles)							
CIMT (mm)	0,80 [0,67-1,01]	0,96 [0,70-1,20]	0,80 [0,65-0,95]	<0,0001	0,77 [0,63-0,93]	0,97 [0,77-1,15]	<0,0001
CRP (mg/L)	5,0 [2,0-8,9]	3,7 [1,5-7,6]	5,4 [2,2-9,2]	0,020	5,0 [1,9-8,6]	5,0 [2,5-9,8]	0,213
GGT (IU/L)	28 [19-44]	32,5 [24,0-47,7]	25,0 [17,0-41,0]	<0,0001	27,0 [19,9-42,0]	29,0 [22,0-50,0]	0,014
Insulin (Mmol/L)	11,7 [6,9-17,4]	10,5 [6,2-15,9]	12,3 [7,3-10,1]	0,023	10,1 [6,2-15,9]	14,8 [9,2-23,2]	<0,0001
Glucose/insulin	0,51 [0,33-0,85]	0,57 [0,34-1,09]	0,52 [0,32-0,77]	0,047	0,49 [0,33-0,81]	0,62 [0,32-0,92]	0,222
HOMA-IR	2,99 [1,66-5,00]	2,7 [1,3-4,2]	3,2 [1,8-5,3]	0,021	2,31 [1,31-3,74]	6,63 [3,52-10,77]	<0,0001
HOMA-β%	110,8 [58,6-185,2]	90,4 [44,7-172,2]	114,0 [65,9-190,4]	0,039	125,3 [78,3-208,7]	59,1 [35,8-121,9]	<0,0001
QUICKI	0,14 [0,13-0,15]	0,14 [0,13-0,16]	0,14 [0,13-0,15]	0,021	0,15 [0,14-0,16]	0,13 [0,12-0,14]	<0,0001
FIRI	2,69 [1,49-4,50]	2,46 [1,17-3,81]	2,93 [1,60-4,76]	0,021	2,08 [1,18-3,37]	5,07 [3,17-9,69]	<0,0001
1/HOMA-IR	0,33 [0,20-0,60]	0,37 [0,24-0,77]	0,31 [0,19-0,56]	0,021	0,43 [0,27-0,76]	0,18 [0,09-0,28]	<0,0001

Abbreviations: CRP, C-reactive protein; CIMT, carotid intima media thickness; FBG, fasting blood glucose; FIRI, fasting insulin resistance index; GGT, γ -glutamyltransferase; HbA1c, glycated haemoglobin; HDL, high density lipoproteins; HOMA-β%, homeostasis model assessment of functional beta-cells; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low density lipoproteins; QUICKI, quantitative insulin sensitivity check index; SD, standard deviation

Table 5.2 Robust correlation of carotid intima media thickness with markers of insulin resistance/sensitivity

Correlates	No diabetes	Diabetes	p-value	Overall						
					FBI	FBG	HOMA-IR	QUICKI	FIRI	Glucose/insulin ratio
FBI	-0,01 (-0,12 to 0,09)	-0,04 (-0,20 to 0,12)	0,80	0,01 (-0,07 to 0,10)	NA					
FBG	0,15 (0,04 to 0,25)	0,16 (0,0 to 0,31)	0,89	0,22 (0,14 to 0,30)	0,00014	NA				
HOMA-IR	0,02 (-0,08 to 0,12)	0,03 (-0,13 to 0,19)	0,91	0,09 (0,01 to 0,18)	<0,0001	0,0015	NA			
QUICKI	0,0 (-0,10 to 0,10)	-0,02 (-0,18 to 0,14)	0,81	-0,06 (-0,14 to 0,03)	0,400	0,00017	0,072	NA		
FIRI	0,02 (-0,08 to 0,12)	0,03 (-0,13 to 0,19)	0,91	0,09 (0,01 to 0,18)	<0,0001	0,0015	>0,999	0,072	NA	
Glucose/insulin ratio	0,06 (-0,04 to 0,17)	0,13 (-0,03 to 0,29)	0,47	0,09 (0,0 to 0,18)	0,340	0,021	0,97	<0,0001	0,97	NA

Abbreviations: FBG, fasting blood glucose; FBI, fasting blood insulin; FIRI, fasting insulin resistance index; HOMA-IR, homeostasis model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index; NA, not applicable

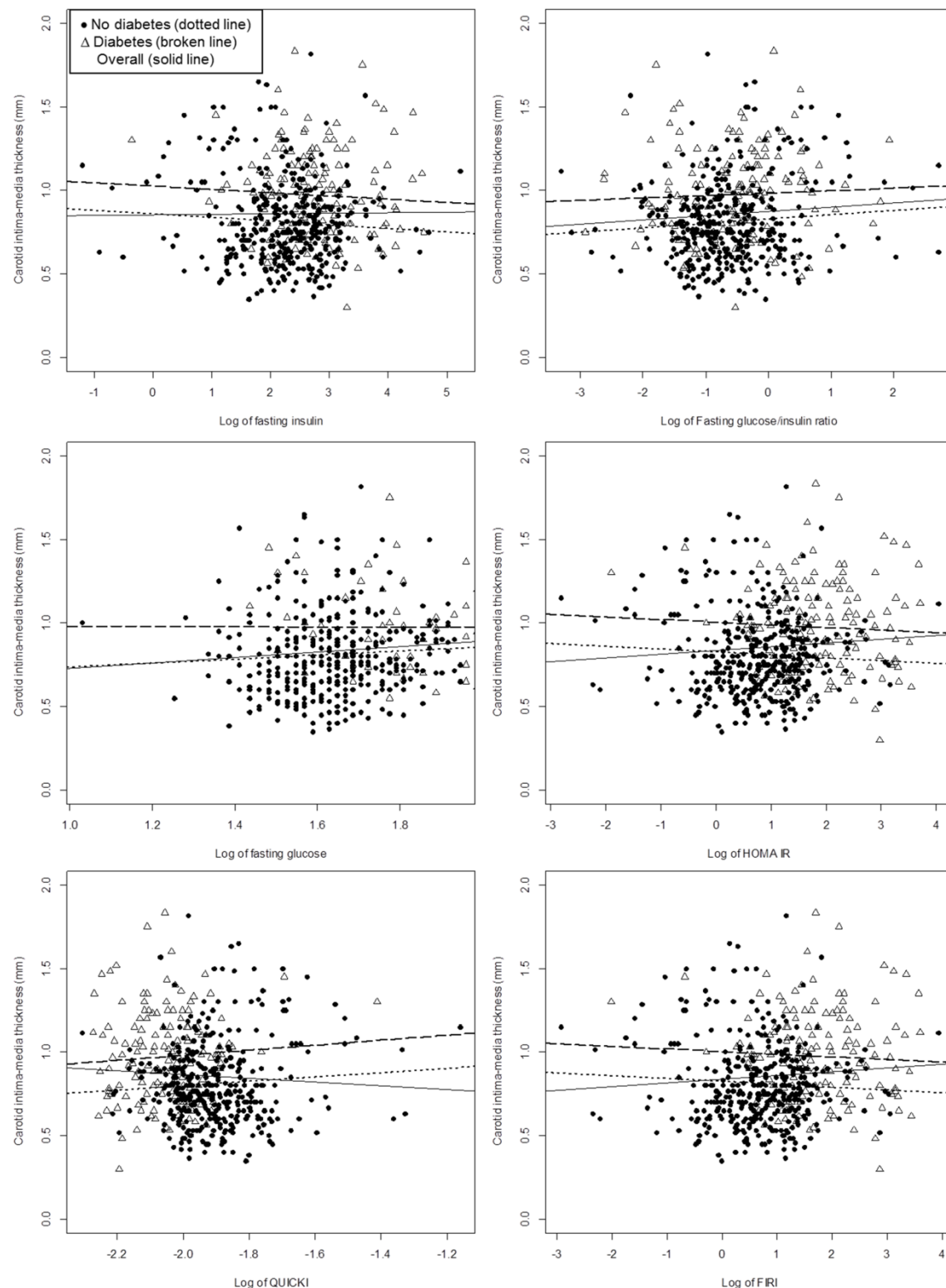


Figure 5.1 Correlations of markers of insulin resistance with carotid intima-media thickness. Participants are grouped by diabetes status with the triangles representing those with diabetes and the solid circles representing those without diabetes. The superimposed regression lines are for the overall sample (solid line) and participants with diabetes (broken line) and without diabetes (dotted lines). Accompanying correlations coefficients are shown in Table 5.2. Abbreviations: FIRI, fasting insulin resistance index; HOMA-IR, homeostasis model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index.

Table 5.3 Regression coefficients from multiple robust linear models for the prediction of carotid intima media thickness by indices of insulin resistance accounting for the potential effect of sex, age, diabetes and adiposity

	Age-Sex-BMI		Diabetes		FBI		FBG		HOMA-IR		QUICKI		FIRI		Glucose/Insulin	
	β	P	β	p	β	p	β	p	β	p	β	p	β	p	β	p
Index	-	-	-	-	-0,020	0,123	0,087	0,042	-0,011	0,359	0,113	0,157	-0,011	0,359	0,026	0,039
Age	0,009	<0,0001	0,008	<0,0001	0,008	<0,0001	0,008	<0,0001	0,008	<0,0001	0,008	<0,0001	0,008	<0,0001	0,008	<0,0001
Sex (men)	0,155	<0,0001	0,155	<0,0001	0,156	<0,0001	0,153	<0,0001	0,155	<0,0001	0,155	<0,0001	0,155	<0,0001	0,156	<0,0001
BMI	0,004	0,003	0,003	0,023	0,004	0,007	0,002	0,054	0,003	0,013	0,004	0,008	0,003	0,013	0,004	0,004
Diabetes	-	-	0,122	<0,0001	0,127	<0,0001	0,072	0,027	0,131	<0,0001	0,135	<0,0001	0,131	<0,0001	0,115	<0,0001
R ²	0,265		0,291		0,295		0,296		0,293		0,294		0,293		0,297	

All indices of insulin resistance are log-transformed.

Abbreviations: BMI, body mass index; FBG, fasting blood glucose; FBI, fasting blood insulin; FIRI, fasting insulin resistance index; HOMA-IR, homeostasis model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index

CHAPTER 6

DISCUSSION/CONCLUSION/RECOMMENDATIONS

6.1 SUMMARY OF FINDINGS

In this study, the genetic results within the mixed-ancestry South African participants uncovered a gene-gene interaction between two previously identified IR loci namely, the *PPARG* and *IRS1* gene variants. The *PPARG* Pro12 allele was strongly associated with IR and its interaction with an additional unfavourable genetic polymorphism, *IRS1* Gly972Arg, conferred an increased risk of type 2 diabetes mellitus. In addition, the *PPARG* His449His variant showed a reduced T2DM risk. Furthermore, the measures of IR in the presence of traditional risk factors, namely age, sex, body mass index and diabetes, were weakly associated with carotid intima media thickness. Based on these results the indices of IR were found not to be a viable marker of CVD risk estimation within this population.

6.2 DISCUSSION AND CONCLUSION

The role of specific SNPs in genes related to IR and T2DM have been well established in Caucasians with studies reporting varying degree of associations in these population groups. Following the global trend observed in other populations, the prevalence of T2DM has also increased at an alarming rate in mixed-ancestry South Africans over the last two decades from 10,8% to 26,4% (Levitt et al., 1999; Erasmus et al., 2012) that is not only attributed to environmental factors but a genetic link as well. Although a vast amount of literature is available on the contribution of genetic factors to the development of IR that consequently leads to T2DM, limited data is available on the South African populations. The interest of this study was to investigate specific candidate genes of IR that possible predispose South Africans, particularly the mixed ancestry population to the early onset and high incidence of type 2 diabetes mellitus.

The etiologic cascade leading to IR, and subsequently T2DM, has been mainly investigated in European Caucasian cohorts with the genome-wide analysis identifying a strong genetic basis that contributes to T2DM susceptibility (Ayub et al., 2014; Dupuis et al., 2010; Povel et al., 2012; Morris et al., 2012; Vioght et al., 2010; Wanatabe, 2010; Brunetti et al., 2014). By far, the *PPARG* Pro12Ala variant has been shown to be a strong candidate gene associated with IR and T2DM with positive replication reported in most studies with an protective effect against type 2 diabetes mellitus (Deeb et al., 1998; Buzzetti et al., 2004; Tönjes et al., 2006; Gouda et al., 2010; Dupuis et al., 2010; Trombetta et al., 2013). In the African population studies, however, the protective allele against T2DM of the

PPARG Pro12Ala variant was rare in Ghanaians but absent in black South Africans from Zulu descent (Danquah et al., 2013; Pirie et al., 2010). However, the *PPARG* Pro12 at risk allele was identified in Ghanaians but no association was evident with IR or T2DM, which could be due to the sample size used in the study (Danquah et al., 2013). In Tunisians no association with T2DM was found with the *PPARG* gene (Turki et al., 2012; Mtiraoui et al., 2012). Similarly, results in mixed-ancestry South Africans showed evidence regarding the association of the risk Pro12 allele in the *PPARG* gene with insulin resistance. However, the *PPARG* Pro12Ala variant did not have a protective effect against the risk of type 2 diabetes mellitus. Although there were differences in the risk allele frequencies when compared to other ethnic populations, the allelic effect associated with the IR and T2DM risk seems consistent in the mixed-ancestry population. Moreover, the combined effect, i.e. gene-gene interaction, of the *PPARG* Pro12Ala and *IRS1* Gly972Arg gene-variants observed in this population has also previously been reported in terms of their effect on IS (Stumvoll et al., 2002) and adiponectin levels (Mousavinasab et al., 2005) associated with IR and diabetes. Furthermore, the genetic prediction of disease is greatly improved if genetic variants with strong effects are identified, either on their own or with other variants, i.e. gene-gene interaction or gene-environment interactions (Janssens & van Duijn, 2008; Luo et al., 2013; Chan et al., 2013).

Regarding the low frequency *PPARG* polymorphisms investigated, only the *PPARG* His449His variant was found in the mixed-ancestry population. As far as is known, this is the first study that demonstrates the T allele of the *PPARG* His449His variant to have a protective effect against the risk of developing diabetes. Although the contribution of rare variants to disease risk is known to be limited, there is strong evidence that they may play a role in complex disease etiology and could possibly have greater genetic effects than common variants. Further development of sequencing technology and methods may increase the possibility to detect rarer complex trait associations that could result in new discoveries related to their underlying effects on disease risk (Manolio et al., 2009; De La Vega et al., 2011). Lack of association was found between the *IRS1* Gly972Arg variant and IR in this population, which is similar to results reported in African Americans (Lei et al., 1999), Mexican Americans (Celi et al., 2000) and Asians (Abate et al., 2003). However, this does not exclude the possibility that other polymorphisms of *IRS1* could have an effect on diabetes.

In conclusion, this current study advances the research knowledge relating to the genetic basis for the development of IR and risk of T2DM in a South African population. Furthermore, the results provide evidence in support of the role of specific mutations of the *PPARG* and *IRS1* genes associated with diabetes through effects on IR, which may explain the high prevalence of T2DM within mixed-

ancestry South Africans. The early detection of subjects with IR at high risk for T2DM could be a valuable factor in confronting the rising prevalence of diabetes already observed in this population.

6.3 LIMITATIONS

Due to the origin of the mixed-ancestry ethnic group, associations identified in this study may be due to population stratification, and this was not taken into account. Therefore, the findings of this study require replication in a larger study involving other homogeneous populations before they can be considered as established in Africa. The candidate gene approach used for association analysis in this study has been proven to be extremely powerful for studying genetic traits of complex diseases like diabetes. However, the disadvantage of this approach is the low replication of results and the limited ability to include all possible causative agents. The power of the study is limited due to the small sample size. This is because most genes contributing to complex disorders confer only a very modest increase in disease risk and to detect these with higher power requires larger sample sizes. Although the simple indices of IR were useful for determining whether a disease was associated with IR, the evaluation of other independent measures of IR could be used to further confirm the findings reported in this population. The number of males versus females is skewed, which is characteristic of epidemiology studies in South African populations.

The strengths of this study are that the participants were well characterised with both fasting and OGTT derived indices used for the assessment of T2DM and insulin resistance. Genotyping was carried out by two independent laboratories. Overall, in spite of the limitations, the study represented a significant advance in the knowledge of the genetic determination of IR and the risk of developing diabetes in an African mixed-ancestry population with a high incidence of type 2 diabetes mellitus.

6.4 RECOMMENDATIONS

The findings of in this study provides a starting point for future studies in other African populations to investigate additional candidate genes as well as genome wide association studies that could possibly identify further functional variants related to IR and T2DM risk. The relative contributions of these genes to T2DM susceptibility could possibly shed more light on the etiological cascade of IR within African populations. Since different ethnic associations of the genetic variations have been associated with IR, the gene-to-gene or gene-to-environment interactions and certain related risk factors may influence the susceptibility to metabolic disorders.

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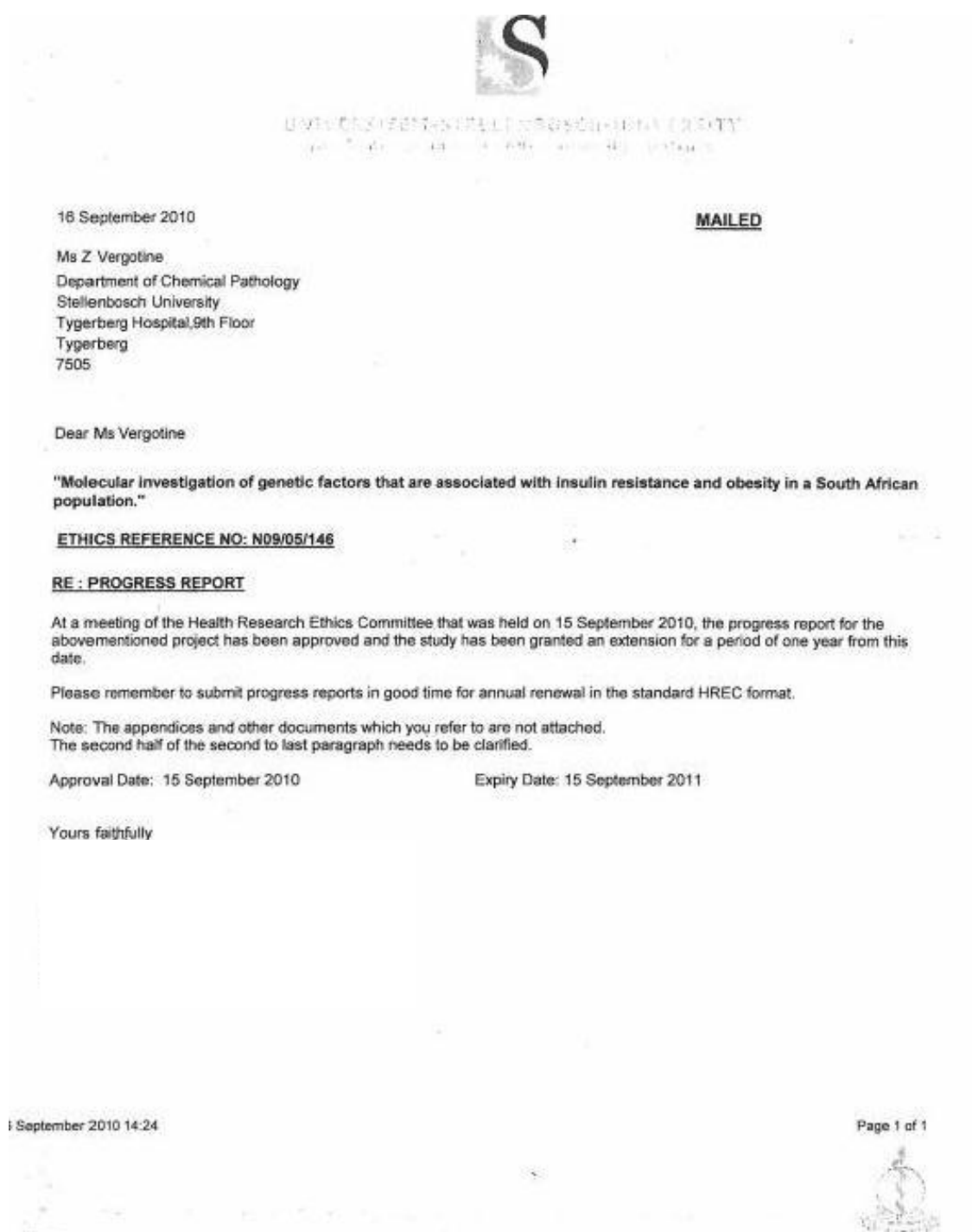
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APPENDICES

Appendix A Ethics approval for the research project



Appendix B Consent form for genetic analysis



THE BELLVILLE SOUTH DIABETES SURVEY CONSENT FORM

Investigators: Prof Matsha (CPUT)
Mr Shafick Hassan (CPUT)
Prof Rajiv Erasmus (University of Stellenbosch)
Mr Soita David Jonah (MTech student)

Address: Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology (CPUT), Bellville Campus
Symphony Way, 7535
Chemical Pathology Department, Faculty of Health Sciences, University of Stellenbosch (Tygerberg Campus), Tygerberg, 7505.

Dear Participant,

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the Committee for Human Research at Cape University of Technology and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

Worldwide new causes of certain diseases or conditions are continuously being discovered by research on the cells and molecules of the body. For research to be carried out on certain diseases it is necessary to first establish the incidence and prevalence of the disease. This project aims to determine the incidence and prevalence of diabetes and glucose intolerance.

Additionally, this project aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Why have you been invited to participate?

The prevalence of diabetes in South Africa is not well documented and few studies have been conducted since 1994. Many subjects with diabetes are unknown to the health service, often because they are not yet diagnosed. In order to assess the magnitude of the problem, you have been approached to participate in this project to determine the incidence of diabetes amongst our adult population.

You have randomly been selected by means of a computer program to participate in the above-mentioned study. Adults of all races, gender, age (between ages 35 and 60 years) and weight will be approached as subjects.

What will your responsibilities be?

The participant will be requested to provide information about his/her medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 10 minutes. Measurement such as weight, height, waist and hip will be done. Fasting Venous Blood will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood will be collected. The blood will be used to determine whether you have diabetes or glucose intolerance. The other tests that will be determined from your blood sample are: Cholesterol and Triglycerides levels. The remainder of the blood sample will be used for genetic and future research studies. The DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

Will you benefit from taking part in this research?

You will be notified of your glucose tolerance state or whether you are diabetic by the medical nurse or doctor. Thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.

In the unlikely event that the research may lead to the development of commercial applications, the participant or the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to my/*the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc.

Are there any risks involved in my taking part in this research?

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse will be present on all occasions. In addition, the research team will be in contact with medical doctors should you need emergency care by a medical doctor.

Who will have access to your medical records?

The participant's identity will be kept confidential throughout. Information will not be associated with the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant.

Some insurance companies may mistakenly assume that taking part in research indicates a

higher risk for disease. Thus no information about you or your family will be shared with such companies.

Will you or your child be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study, but your transport, if required will be covered for each study visit. It is envisaged that you may be hungry since you would have come fasting, therefore, biscuits or fruit will be provided. There will be no costs involved for you if you take part in the project.

Is there anything else that you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study.

You will receive a copy of this information and consent form for your own records if it is requested

DECLARATION BY PARTICIPANT:

I declare that:

I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is voluntary and I have not been pressurized to take part.

I may choose to withdraw from the study at any time and will not be penalized or prejudiced in any way.

I may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan as agreed to.

I also consent that my blood may be:

☐ **Used and stored for future research studies**

☐ **Used and discarded**

Signed at (*place*) on (*date*) 2007

Signature of participant_____ **Signature of witness**_____

DECLARATION BY THE INVESTIGATOR

I (*name*) declare that:

I explained the information in this document to (*Names of participant*).....

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understand all aspects of the research, as discussed above

Signed at (*place*) on (*date*) 2007.

Signature of investigator _____ Signature of witness _____

Appendix C Questionnaire of lifestyle factors



Progressive research on risk factors of type 2 diabetes and cardiovascular diseases in South Africa.

Principal Investigator: Prof Tandi Matsha

Name of Interviewer:

Date of Interview:/...../.....

Ref No

To the respondent:

Thank you very much for your willingness to participate in the completion of this questionnaire. The information obtained on this questionnaire will provide us with information on all the possible health, family, life style and dietary risk factors within your house hold that might influence the development of diabetes. This is because many health conditions develop slowly over time yet could be prevented if diagnosed early or if pre determined. This questionnaire therefore aims at getting information which may be used to determine the extent of diabetes and those likely to develop diabetes in the future. The questionnaire should not take long and we hope you find it interesting and enjoyable. All answers provided will be treated as confidential and anonymous.

Note

No special knowledge is needed to fill this questionnaire. Please feel free to ask for clarification if needed.

Postal Address: -----

Residential address: -----

Telephone OR
Cell phone Contacts: -----

A. PERSONAL DATA

Instructions:

Please complete the following general information about yourself by ticking in the box next to the appropriate answers. Please take your time and read through questions carefully.

1. What is your date of birth? ☐
2. What is your gender?
Male ☐ Female ☐ ☐
3. What is your marital status?
Married ☐ Single ☐ Widowed ☐
Divorced ☐ Other ☐ ☐
4. How would you describe your self?
Black ☐ White ☐
Colored ☐ Asian ☐ ☐
5. What is the highest level of education you have completed?
(a) Primary School or less ☐
(b) High School (Not Completed) ☐
(c) High School graduate ☐
(d) College Or Technical College (Not Completed) ☐
(e) College or Technical College Graduate ☐
(f) University or technikon (Not Completed) ☐
(g) University or technikon graduate ☐
6. What is your Profession/Occupation?
Please state..... ☐
- 7 How long have you been living in Bellville South?
Less than 6 Months ☐ Less than 1 Year ☐
1-5 Years ☐ 5 years and above ☐ ☐

B. FAMILY HEALTH HISTORY

Instructions:

The following questions will tell us about your family health history. Please complete all the questions by placing a tick next to the appropriate answer or writing in the answer.

8. Are you currently on any medication?	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
9. If Yes, Please list.....			<input type="checkbox"/>
10. Have you ever been told that you have diabetes?			
Yes <input type="checkbox"/>	No <input type="checkbox"/>		<input type="checkbox"/>
11. Have any of the following in your family ever had or are being treated for diabetes?			
(a) Mother	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(b) Father	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(c) Sister(s)	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(d) Brother(s)	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(e) Husband/Wife	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(f) Children	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(g) Grandchildren	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>

12. Have any of the following extended family members ever suffered or are suffering from diabetes?

(a) Paternal (Fathers Side)

(i) Uncles

Yes ☐

No ☐

☐

(ii) Aunties

Yes ☐

No ☐

☐

(iii) Grandparents

Yes ☐

No ☐

☐

(b) Maternal (mothers Side)

(i) Uncles

Yes ☐

No ☐

☐

(ii) Aunties

Yes ☐

No ☐

☐

(iii) Grand Parents

Yes ☐

No ☐

☐

13. Have you or any of the following ever been treated for heart problems?

(a) Yourself

Yes ☐

No ☐

☐

(b) Spouse

Yes ☐

No ☐

☐

(c) Mother

Yes ☐

No ☐

☐

(d) Father

Yes ☐

No ☐

☐

(e) Children

Yes ☐

No ☐

☐

(f) Grandparents	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(g) Sisters	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(h) Brothers	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>

14. Have any of the following ever been treated for High Blood pressure?

(a) Yourself	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(b) Spouse	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(c) Mother	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(d) Father	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(e) Children	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(f) Grandparents	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(g) Sisters	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(h) Brothers	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>

15. Did either of your natural parents ever die of a heart attack?

(a) Before the age of 60?	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(b) After the age of 60?	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>

16. Have you or any of the following ever been treated for High Cholesterol?

(a) Yourself	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(b) Spouse	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(c) Mother	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(d) Father	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(e) Children	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(f) Grandchildren	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(g) Brother	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>
(h) Sisters	Yes <input type="checkbox"/>	No <input type="checkbox"/>	<input type="checkbox"/>

E. ALCOHOL USE

22. Have you ever consumed any alcoholic drinks (Wine, Beer, and Spirits)?

Yes ☐

No ☐

☐

23. Do you still consume alcoholic drinks?

Yes ☐

No ☐

☐

24. If you consume or consumed alcohol, how old were you when you first started drinking?

Please state.years

☐

25. If you stopped, how old were you when you stopped drinking?

Please state.

☐

26. Which type of alcohol do you or did you drink?

Wine ☐

Beer ☐

Spirits ☐

☐ Others, please indicate.....

☐

27. When you drink or drank alcoholic drinks, how many drinks or glasses do you or did you consume daily? Indicate the number.....

☐

28. How many days a week do you or did you consume alcohol?

1-2 ☐

3-4 ☐

5-6 ☐

every day ☐

☐

29. Have you or did you ever feel you should cut down your drinking?

Yes ☐

No ☐

☐

30. Have people ever annoyed you by criticizing your drinking?

Yes ☐

No ☐

☐

31. Have you ever felt bad about your drinking?

Yes ☐

No ☐

☐

32. Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover (Eye Opener)

Yes ☐

No ☐

☐

F. BODY MEASUREMENTS. Interviewers Name.....

33. Weight and Height.

Body Weight (kg)			
Body height (cm)			
Body Mass Index			

34. CALLIPERS MEASUREMENTS

Biceps	1 (cm)		
Biceps	2 (cm)		
Biceps	3 (cm)		
Total			
Triceps	1 (cm)		
Triceps	2 (cm)		
Triceps	3 (cm)		
Total			
Sub-Scapular	1 (cm)		
Sub-Scapular	2 (cm)		
Sub-Scapular	3 (cm)		
Total			
Suprailiac	1 (cm)		
Supra-iliac	2 (cm)		
Supra- Iliac	3 (cm)		
Total			

35. CIRCUMFERENCE MEASUREMENTS

Waist Circumference	1 (cm)		
Waist Circumference	2 (cm)		
Waist Circumference	3 (cm)		
Total			

--	--	--

36.

Hip Circumference	(cm)		
Hip Circumference	(cm)		
Hip Circumference	(cm)		
Total		(cm)	

--	--	--

37. BLOOD PRESSURE MEASUREMENTS. Interviewers Name.....

Systolic Pressure	1 (mmHg)		
Systolic Pressure	2 (mmHg)		
Systolic Pressure	3 (mmHg)		

--	--	--

Diastolic Pressure	1 (mmHg)		
Diastolic Pressure	2 (mmHg)		
Diastolic Pressure	3 (mmHg)		

--	--	--

Pulse 1	(Beat per Minute)			
Pulse 2	(Beat per Minute)			
Pulse 3	(Beat per Minute)			

--	--	--

G. BLOOD ANALYSIS. Interviewers Name.....

38. (a) Fasting State Measurements

(i) Did subject eat this morning?..... If yes state time.....

--	--

(ii) When did subject eat the last meal last evening?.....

--	--

(iii) Please indicate the time when fasting blood taken.....

--	--

(iv) Please indicate the time when Glucose was given.....

--	--

Glucose	mmol/l		
Cholesterol	mmol/l (L=1, N=2, H=3)		
Triglycerides	mmol/l (L=1, N=2, H=3)		

(b) Post Prandial Measurements

--	--

Please indicate time when post prandial blood was taken.....

Glucose	mmol/l		
Cholesterol	mmol/l		
Triglycerides	mmol/l		

H. Researchers Check list.

Please make a tick or cross against each of the following questions

- | | |
|---|--------------------------|
| 1. Were all personal data questions answered? | <input type="checkbox"/> |
| 2. Were all Family Health History questions answered? | <input type="checkbox"/> |
| 3. Were all questions on Tobacco use answered? | <input type="checkbox"/> |
| 4. Were all questions on Alcohol use answered? | <input type="checkbox"/> |
| 5. Were all dietary questions answered? | <input type="checkbox"/> |
| 6. Were all body measurements carried out? | <input type="checkbox"/> |
| 7. | <input type="checkbox"/> |
| (a) Was fasting blood taken? | <input type="checkbox"/> |
| (b) Was Glucose Given? | <input type="checkbox"/> |
| 8. Was Post Pradial Blood taken? | <input type="checkbox"/> |
| 9. Was B.P Taken | <input type="checkbox"/> |

Appendix D Principles and performance characteristics of assays used

Assay/Analyte	Principle/CAT #	CV	Linearity/ Measuring range
Plasma glucose	Glucose is oxidised by glucose oxidase to produce gluconate and hydrogen peroxide (HP). The HP then reacts with 4 amino-antipyrine (4-AAP) and phenol in the presence of peroxidase to yield a red quinoneimine dye. The absorbance of the dye at 505 nm is proportional to the concentration of glucose in the sample.	3,0	0,11-41,6 Mmol/L
Insulin	Microparticle enzyme immunoassay.	2,4	1,39-6945 pmol/L
High sensitive (CRP)	C-reactive protein (CRP) agglutinates with latex particles coated with monoclonal antibodies against CRP. The precipitate is measured turbidimetrically.	2,1	0,15-20,0 mg/L
Glycosylated haemoglobin (HbA1c)	Total haemoglobin (Hb) is determined in an alkaline medium based on the formation of a brownish-green chromophore whose colour intensity corresponds to the concentration of Hb in the sample and is determined by the increase in absorbance at 552 nm. HbA1c is measured using monoclonal antibodies that bind the β -N-terminal fragments of HbA1c. The final percentage HbA1c [HbA1c(%)] is computed from the ratio of both concentrations [HbA1c(%) = (HbA1c/Hb) \times 87,6 + 2,27].		
Alanine aminotransferase (ALT)	Alanine catalyses the reaction between L-alanine and oxoglutarate forming pyruvate and glutamate. Lactate dehydrogenase then catalyses the reduction of the pyruvate forming L-lactate and nicotinamide adenine dinucleotide (NAD ⁺). The rate at which the NADH is oxidised is directly proportional to the catalytic ALT activity and is determined by measuring the decrease in absorbance at 340 nm.	3,0	5-700 U/L
γ -glutamyl transferase (GGT)	GGT-catalysed transfers of an γ -glutamyl group from L- γ -glutamyl-3-carboxy-4-nitroanilide to glycylglycine liberate 5-amino-2-nitrobenzoate at a rate proportional to the GGT activity in the sample and are determined by measuring the increase in absorbance at 409 nm.	1,3	5-720 U/L

Total cholesterol	Cholesterol esterase (CE) hydrolyses cholesterol esters to form free cholesterol and fatty acids. Cholesterol oxidase (CHOD) then catalyses the oxidation of cholesterol to form cholest-4-ene-3-one and H ₂ O ₂ . Hydrogen peroxide leads to the oxidative coupling of phenol and 4-amino-antipyrine (4-AAP) to form a red-coloured quinoneimine chromogen whose intensity is directly proportional to the cholesterol concentration.	2,5	0,25-20,7 Mmol/L
High-density lipoprotein (HDL)	Polyethylene glycol (PEG)-modified cholesterol esterase catalyses the breakdown of HDL-cholesterol (HDL-c) esters to free cholesterol and fatty acids. The free cholesterol is then oxidised by PEG-cholesterol oxidase to Δ^4 -cholestenone and H ₂ O ₂ , which oxidises 4-AAP forming a blue quinoneimine dye, whose intensity correlates with the HDL-c concentration and is determined by measuring the increase in absorbance at 583 nm.	1,5	0,08-7,2 Mmol/L
Triglycerides (TG)	Lipoprotein lipase hydrolyses triglycerides (TG), producing glycerol, which is then phosphorylated by adenosine triphosphate (ATP) to glycerol-3-phosphate (G-3-P) in a glycerol kinase catalysed reaction. Oxidation of G-3-P in the presence of glycerol phosphate oxidase yields H ₂ O ₂ , which drives the reaction of 4-chlorophenol and 4-aminophenazone forming a red-coloured chromogen measured at 512 nm. The increase in absorbance corresponds to the concentration of TG in the sample.	1,3	0,1-10,0 Mmol/L
Creatinine	Creatinine reacts with picrate forming a yellow-orange complex at a rate that is proportional to the creatinine concentration in the specimen.	1,3	15-2200 μ mol/L

Abbreviations: CV, Coefficient of Variation (%); CAT#, Catalogue reference number

Appendix E Primers designed

Polymorphism	Chromosome Location	Primer Sequence	Tm	GC %	Product size
<i>PPARG</i> Pro12Ala rs1801282	3:12351626	F: GAGCGCCCAGATGAGATTAC=20 R: CTGGATCTTGTCTTGTGAATGG=22	59,8 59,6	55 45,5	300bp
<i>PPARG</i> Pro115Gln rs1800571	3:12381349	F:TGAAGGTGTACTATGGTGGCTTGC=24 R:CCACAGACACGACATTCAATTGCC=24	58,8 58,5	50 50	205bp
<i>PPARG</i> His447His rs3856806	3:12434058	F:GATGAGTTGCTTGGTAGAGCTG=22 R:TGGAAGAAGGGAATGTTGG=20	59,1 59,9	50 45	386bp
<i>PPARG</i> Val290Met rs72551362	3:12416836	F:TTGCAGCCATTCGTTATCTATG=22 R:TCGGATATGAGAACCCCATC=20	60,1 59,7	40,9 50	299bp
<i>PPARG</i> Phe388Leu rs72551363	3:12417048	F:TGTGCAGGAGATCACAGAGTATGC=24 R:CCATCATCCCAACCTCTTTC=20	63,9 61,6	50 55	357bp
<i>PPARG</i> Arg397Cys rs72551364	3:12433900	F:CAGATGAGTTGCTTGGTAGAGC=22 R:TCCGTGACAATCTGTCTGAGG=21	59,1 61,3	50 52,4	257bp
<i>IRS1</i> Gly972Arg rs1801278	2:226795828	F:CAGCTCCCAGAGAGGAAGAG=20 R:TGTTTCGCATGTCAGCATAGC=20	59,3 61,0	60 50	266bp

Abbreviations: F, Forward primer; R, Reverse primer; Tm, melting temperature in degree Celsius; GC, rich template guanine cytosine content in percentage; bp, base pairs, ***PPARG***; peroxisome proliferator-activated receptor gamma, ***IRS1***; insulin receptor substrate 1